Profiling Inter-Individual Differences in Human Cellular Signaling

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'' Dance of the Clock Gene Proteins''

Painted by Julie Newdoll

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Moja najvacsia vdaka patri trom najdolezitejsim ludom mojho zivota : Borisovi, mamine a braskovi Palinovi. Ich laska a neustala podpora ma posuvala vpred a bez nich by som to nikdy nedokazala.

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Summary

The human body contains hundreds of cell types, which are specialized to form various tissues and organs with a particular function. The communication between these cells is a key aspect of any multi-cellular biological system and is mediated by specialized signal transduction cascades. Such cascades generally begin by binding of extracellular ligands such as neurotransmitters, growth factors or cytokines to a specific cell surface receptor, or by the activation of an intracellular sensor. Its signal is propagated, regulated, and amplified by other proteins, often *via* protein phosphorylation. Ultimately, a final effector molecule -- itself regulated by phosphorylation -- binds to conserved elements in many promoters to control a variety of processes. Although the cell responds to a plethora of signals and environmental conditions in many different ways, there is only a small set of signal transduction cascades, each conserved among many different cell types.

These highly conserved signaling pathways open the possibility to study human inter-individual differences in neurologically and physiologically relevant processes using accessible primary tissues, i.e. skin fibroblasts. The idea of using skin as a proxy for what happens in the brain is not as farfetched as it seems. For example, many studies showed that not only the neurons of the central clock in the brain but also many peripheral tissues possess circadian clocks. The circadian signaling pathway is one crucial transduction pathway, which is responsible for the production and maintenance of daily rhythms in physiology, mood and cognition. Therefore, this high level of homology in signal transduction pathways provides a possibility to study human differences at a cellular level in peripheral tissues.

The circadian properties (e.g. Circadian period, phase or amplitude) of human beings differ widely in the population and several studies showed that the daily behavior has a significant genetic component. On the top of this, recent investigations showed that the *in vitro* circadian period length of human skin fibroblasts measured through a lentiviral reporter system correlates with the human physiological period length and is in turn directly predictive of human diurnal behavior. All these findings suggested that signaling outputs obtained from the human primary fibroblasts could in the future serve as an excellent system to determine the genetic basis of human inter-individual differences, and formed the basis of my PhD project.

The first part of this thesis focuses on the study of inter-individual differences of human circadian properties. The primary aim of this study was to establish a technology which would facilitate the expression quantitative trait mapping of genetic loci that influence circadian behavior within the human population. Our laboratory uses a lentivirus cell-based reporter construct containing mouse Bmal1 promoter, the firefly luciferase coding region and the Bmal1 3'UTR, flanked by the long terminal repeats. Immortalized lymphoblastoid cell lines as well as human umbilical cord fibroblasts derived from the healthy individuals, represent sufficient cell libraries required for the linkage and/or association mapping of a causative genetic modifier. To our big disappointment, lymphoblastoid cells revealed almost no circadian oscillations and although umbilical cord fibroblasts seem to have a modest circadian rhythmicity, the amplitude of these rhythms was extremely low and quickly damping.

Besides the circadian signaling pathway, other non-circadian transduction cascades are key regulators of the multilevel cellular communication responsible for many essential biological processes. Technologies similar to those developed to study the human circadian rhythms variation could be equally applied to these non-circadian signaling cascades. Therefore, the second part of the thesis deals with a development of lentivirus – based technologies that allow determination of human inter-individual variation in gene expression of major signal transduction pathways. By using this system, surprisingly large inter-individual differences in the expression pattern of CREB, Elk1 and CHOP signaling pathways have been discovered. The existence of these variations was confirmed by genome-wide gene expression analysis, uncovering differences in transcription from genes known to be activated by these pathways. Moreover, at least on the cellular level these differences have functional relevance because they also translate into differences in cellular toxicity and efficacy of pathway-specific drugs. To demonstrate the broader physiological relevance of these findings, I was able to correlate reporter-detected inter-individual differences in human fibroblast CREB signaling with actual suppression of the hormone melatonin by light in the same human subjects, a process known to be CREB-dependent from rodent studies.

The third and last part of the thesis describes the broader impact of the developed technologies. Independent proliferation, avoidance of apoptosis and unlimited replicative potential -- the hallmarks of tumorigenesis -- are signatures for altered functions of signal transduction cascades. Many studies have already shown that balance and integration among

these signals varies among different tumors. Knowledge about their signaling cascade function could serve as an important marker for successful medication. Therefore, I applied our novel lentiviral reporter system to trace the behavior of signaling pathways within growing tumors in living mice. An interesting kinetic pattern, with possible *in vivo* activation of some signaling pathways during tumor progression was obtained. As cancer development is an extremely complex process involving multilevel communication between many cells, understanding the basis of altered signal transduction might help to improve existing drug treatment strategies and bring more insights into the variability of cancer pathogenesis among human populations.

At a cellular level, nearly all drugs act upon regulatory proteins of signaling cascades that transmit information from the cell surface in order to elicit cytoplasmic or nuclear changes. Therefore, the widespread problem of inter-individual variations in responses to many classes of drugs actually begins here: due to genetic variation, the expression or structure of signaling cascade proteins can differ from one person to another. Understanding the cellular determinants of these differences is a fundamental question for pharmacology, and is the basis of "personalized medicine." In the course of my thesis work, I have developed a novel high-throughput technology that is capable of measuring differences in the output of several signaling cascades between human subjects. This system could now be applied in the testing of many pharmaceutical compounds directly in human primary fibroblasts derived from multiple individuals. Understanding patterns of gene expression variation among human individuals will provide powerful insights into the molecular basis of phenotypic variation and interpretation of differences in the expression patterns in disease.

Zusammenfassung

Der menschliche Körper besteht aus hunderten verschiedener Zelltypen, die Gewebe und Organe mit spezifischen Funktionen bilden. In einem solchen multizellulären System ist die Verständigung zwischen den Zellen von zentraler Bedeutung und erfolgt mittels spezialisierter Signaltransduktionskaskaden. Im allgemeinen beginnen diese Sinalwege wenn Neurotransmitter, Wachstumsfaktoren oder Zytokine an spezifische Rezeptoren auf der Zelloberfläche binden oder intrazelluläre Sensoren aktiviert werden. Dieses Signal wird dann von anderen Proteinen – oft durch Phosphorylierung derselben – weitergeleitet, reguliert und verstärkt. Schliesslich bindet am anderen Ende der Kaskade ein Effektormolekül, selbst durch Phosphorylierungsänderungen reguliert, an konservierte Motive im Promotorbereich zahlreicher Gene und kontrolliert so diverse Prozesse. Im Vergleich zu der grossen Anzahl verschiedener Signale und Umweltreize auf die die Zelle in jeweils spezifischer Weise reagieren muss, gibt es nur wenige Signaltransduktionskaskaden, die dazu benötigt werden. Hinzu kommt, dass diese Kaskaden auch zwischen verschiedenen Zelltypen keine wesentliche Unterschiede aufzeigen

Hier bietet sich die Chance diese prinzipielle Ähnlichkeit zwischen verschiedenen Zelltypen auszunutzen, um interindividuelle Unterschiede neurologisch und physiologisch relevanter Prozesse beim Menschen in leicht zugänglichen Hautzellen beziehungsweise Fibroblasten zu untersuchen. Die Strategie, die Haut als Model für Vorgänge im Gehirn zu benutzen, hat sich schon in anderem Kontext als erfolgreich erwiesen. Beispielsweise haben schon viele Studien gezeigt, dass nicht nur die Neuronen der zentralen zirkadianen Uhr im Gehirn die Zeit messen können, sondern auch viele periphere Gewebe die gleiche Eigenschaft haben. Dieser zirkadiane Signalweg generiert die 24-Stunden Rhythmen der menschlichen Physiologie und Psychologie. Das hohe Mass an Übereinstimmung der zellulären Signaltransduktionswegen ermöglicht es, inter-individuelle Unterschiede zwischen Menschen auf zellulärer Ebene in peripheren Geweben zu untersuchen.

Die Eigenschaften der zirkadianen Uhr des Menschen (beispielsweise Periodenlänge, Phasenlage oder Amplitude) unterscheiden sich stark zwischen Individuen. Mehrere Studien geben Grund zur Annahmen, dass diese Unterschiede zu einem wesentlichen Anteil genetisch bedingt sind. Neueste Resultate, die mit Hilfe eines lentiviralen Reporter-Systems gewonnen wurden, zeigen sogar, dass die Periodenlänge der zirkadianen Uhr in Fibroblastenkulturen in

vitro mit der in vivo gemessenen Periodenlänge übereinstimmt und somit das diurnale Verhalten beim Menschen spiegeln kann. Die genannten Ergebnisse deuten darauf hin, dass die Endpunkte der Signalwege in humanen Fibroblastenkulturen zukünftig ein exzellentes System zur Erforschung inter-individueller Unterschiede beim Menschen darstellen könnten. Dies ist die Eingangshypothese meines Promotionsprojektes.

Der erste Teil dieser Arbeit widmet sich der Erforschung inter-individueller Unterschiede der zirkadianen Uhr des Menschen. Das vorrangige Ziel dieser Studie war die Etablierung einer Technologie zur Erfassung von *expression quantitative trait mapping* genomischer Loci (eQTL), die die Eigenschaften der Inneren Uhr des Menschen beeinflussen. Immortalisierte lymphoblastoide Zelllinien und Nabelschnurfibroblasten von gesunden Freiwilligen sind in genügend grosser Zahl vorhanden, um *linkage* oder *association mapping* kausativer genetischer Modifikatoren durchzuführen. Allerdings hat sich zu unserer grossen Enttäuschung herausgestellt, dass lymphoblastoide Zellen praktisch keinen messbaren zirkadianen Rhythmus haben und obwohl Nabelschnurfibroblasten zumindest messbar rhythmisch sind, auch hier die Amplitude dieser Oszillationen extrem niedrig war und zudem schnell gedämpft wurde.

Neben dem zirkadianen Signaltransduktionsweg gibt es andere, nicht-zirkadiane Signaltransduktionskaskaden, die Schlüsselpositionen in der Regulation der vielschichtiger zellulären Kommunikation innehaben. Sie sind für viele essentielle biologische Prozesse verantwortlich. Ähnliche, wie die für die Erforschung der Variabilität der Inneren Uhr des Menschen entwickelte Technologien können auch auf die nicht-zirkadianen Signaltransduktionskaskaden angewendet werden. Im zweiten Teil der vorliegenden Arbeit wurde daher eine auf Lentiviren basierende Technologie entwickelt, die es erlaubt, die inter-individuellen Unterschiede auf Ebene der Genexpression der wesentlichen Signaltransduktionskaskaden beim Menschen zu erfassen. In der Tat konnten mit Hilfe dieses Systems überraschend grosse inter-individuelle Unterschiede in den CREB, Elk1, und CHOP Signalwegen gefunden werden. Dieselben Unterschiede waren auch in der Expression von Zielgenen der jeweiligen Signalwege wieder zu finden. Zumindest in Zellen sind diese inter-individuellen Variationen auch funktional relevant, denn Signalweg spezifische Medikamente haben differentielle Effekte und zeigen auch eine unterschiedliche Zelltoxizität. Um dies in einen weiteren, physiologisch bedeutungsvollen Zusammenhang zu stellen, habe ich die inter-individuellen Unterschiede in menschlichen Fibroblastenkulturen im CREB Signalweg mit der

lichtinduzierten Melatoninsuppression, ein CREB-abhängiger Prozess, in denselben Menschen korreliert.

Im dritten und letzten Teil dieser Arbeit gehe ich auf das immense Potenzial der von mir entwickelten Technologien ein. Ungesteuerte Vermehrung, Überwindung von Apoptose und unbeschränkte Vermehrung – die wichtigsten Merkmale der Tumorgenese – sind Anzeichen einer Veränderung in verschiedensten Signaltransduktionswegen. Dass die Balance zwischen den einzelnen Signalkaskaden, sowie die Integration der Signale in Tumoren verändert sein kann, ist eine vielfach publizierte Beobachtung. Das Wissen um pathologische Veränderung in diesen Signalwegen könnte daher als ein wichtiger Marker für die erfolgreiche Behandlung genutzt werden. Deshalb habe ich das von mir entwickelte lentivirale Reportersystem genutzt, um die Veränderungen in der Aktivierung von Sinaltransduktionswegen in wachsenden Tumoren im Tiermodell zu untersuchen. Dabei faszinierte besonders die Kinetik der Aktivierungsmuster einiger Signalwege während der Tumorentwicklung. Gerade weil die Krebsentstehung ein extrem komplexer Prozess unter Einbeziehung multiplen Ebenen der Zell-Zell Kommunikation ist, könnte das bessere Verständnis veränderter Signaltransduktionswege in Krebszellen dabei helfen die inter-individuelle Variabilität in der Krebs Pathogenese beim Menschen zu verstehen und die existierenden Behandlungsmethoden zu verbessern.

Auf Ebene der Zelle wirken nahezu alle Medikamente auf regulierende Proteine innerhalb von Signaltransduktionskaskaden, die Information von der Zellmembran aufnehmen und eine Reaktion im Zytoplasma oder Nukleus der Zelle hervorrufen. Dies ist auch ein wesentlicher Grund für inter-individuelle Unterschiede in der Wirkung vieler Medikamente: Aufgrund genetischer Unterschiede kann die Expression oder aber auch die Struktur von Proteinen einer Signaltransduktionskaskade zwischen Menschen variieren. Die zellulären Determinanten, die die Grundlage für diese Unterschiede bilden, zu verstehen ist eine fundamentale Aufgabe der Pharmakologie und ist zum Ausgangspunkt für die personalisierte Medizin geworden. Während meiner Promotionsarbeit habe ich eine neue Hochdurchsatztechnologie zur Messung von inter-individuellen Unterschieden im Ausgangssignal verschiedener Signaltransduktionskaskaden etabliert. Dieses System kann nun unter Anderem zur Charakterisierung verschiedenster pharmakologisch aktiver Substanzen direkt in humanen Fibroblastenkulturen Anwendung finden. Die individuell verschiedenen Genexpressionsmuster zu erfassen und so die molekularen Ursachen von phänotypischen Unterschieden zwischen Individuen zu verstehen, wird

weitreichende Einsichten, auch in die pathologischen Veränderungen dieser Muster bei Erkrankungen, ermöglichen.

CHAPTER 1

General Introduction

1.1 Time and Clock

The nature and meaning of time has always been fascinating for people of all ages. While ancient Greek philosophers such as Aristotle believed that the universe is born as well as dies in endless cycles of infinite time, much later Sir Isaac Newton defined both space and time as absolute and uniform. Coupling of the Newtonian, Kant's and Einstein's theories by the theoretical physicist Stephen Hawking built up today's modern and unique view of the generally accepted four – dimensional space-time where many relative rates of "local clocks" depend on their relative motion.

As the time of the universe goes by, the most authoritative physical oscillation emphasizing our relationship with time is the day-night cycle ruling and organizing our lives in a 24 – hour schedules. But … how do we perceive or measure time? As early as 3500 BC, the Sumerians and the Egyptians are thought to be the first who developed the simple shadow, sundial and later water clock. With time, clocks became more and more precise time – keeping indicators that measure and keep track of the daytime. However, having modern electric clocks in the pocket is not enough to coordinate our day – night cycle activities. There must be some endogenous, more sophisticated mechanism regulating nearly all aspects of our physiology, metabolism and behavior.

It has been assumed for a long time, that circadian clocks - capable of being entrained to the external environment, are an evolutionary adaptation to life in a rotating planet. Nevertheless, a proof of their existence was successfully ignored for a long time. The first documentation of a circadian process can be dated to the 4^{th} century, where Androsthenes, admiral of Alexander the Great, described daily rhythms of leaves opening and closing. Unfortunately, this occurrence was assumed to be a direct response to the external stimuli and the discovery of the circadian rhythms was delayed until the year 1729. A French astronomer Jean Jacques d'Ortous deMairan went far beyond noticing the leaf movement. A simple experiment on the plant *Mimosa* revealed the

existence of an internal circadian rhythm. deMairan showed that even after the exposing the plant to the conditions of constant darkness leaves still possess their rhythmic behavior. deMairan also hypothesized that other factors (e.g. Temperature) might have a significant effect on the circadian rhythmicity. About a century later, a Swiss botanist Alphonse de Candolle added minor, but extremely valuable information into the science of circadian rhythms. Following deMairan's observations in *Mimosa*, he noticed that the period of the leaf's rhythms is starting a little bit earlier with each day. This phenomenon was later defined as a free running period of the circadian rhythms.

Given the origin of circadian biology, it is not surprising that the question of endogenous timing attracted the interest of many scientists from the field of neuroscience, molecular biology, genetics and physics and thereby propelled this scientific field forward.

1.2 Mammalian architecture of circadian clocks

Enormous advances in molecular biology and genetics during the last few decades helped scientists to crack the nature and role of biological rhythms, especially endogenously generated circadian rhythms. The term circadian originates from the Latin *circa diem* what literally means 'about a day'. Nearly all inhabitants of Earth have developed this daily time - keeping system, which helps them to adapt better to external 24 - hour cycles.

In mammals, a broad spectrum of physiology such as sleep – wake cycles, cognitive performance, cardiovascular system, hormone excretion, digestion and detoxification is orchestrated by the circadian system. The architecture of this system is generally organized in a hierarchy of oscillators with the central oscillator at the top. A separate input pathway delivers signals from the external environment to the central pacemaker, which in turn is able to receive and integrate incoming information. The physiology of the whole living system is then affected via output pathways controlled by this master oscillator. The three main blocks of the circadian system - input, central oscillator as well as output pathways, are the topic of the following chapters (Figure 1).

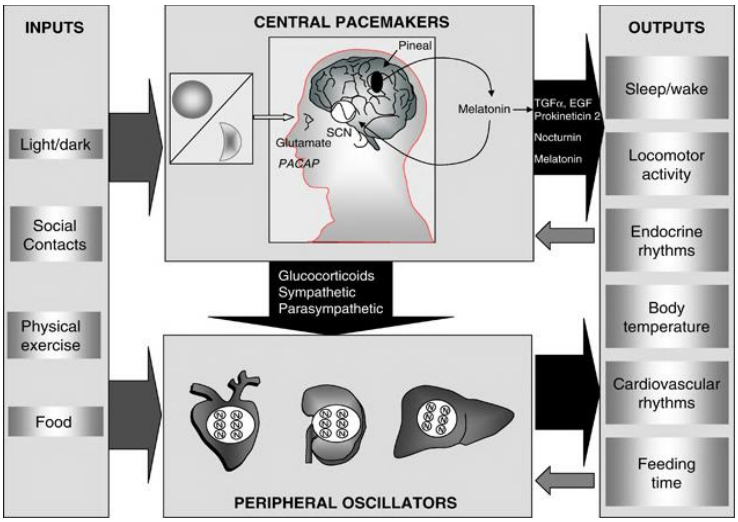


Figure 1 Architecture of the mammalian circadian system. Environmental input cues such as light, food, physical exercise etc. entrain the phase of the central oscillator, located in the SCN of the brain hypothalamus. The central pacemaker is then driving the rhythmicity in other parts of the brain as well as in peripheral tissues via various neural and humoral output signals. The central pacemaker and peripheral tissues are responsible for the daily rhythms in human physiology and behavior. Reproduced from Garaulet et al., 2010 [1].

1.2.1 Suprachiasmatic nucleus – function and structure

A chief oscillator of mammalian rhythmicity, able to generate and sustain the circadian oscillations is located in the suprachiasmatic nuclei (SCN) - in the rostroventral hypothalamus [2]. This tiny little paired structure just above an optic nerve contains approximately 16 000 neurons and became an object of intense research in the mid-1970s.

In 1972, Robert Moore's studies revealed that external photic information is communicated to the brain via the main projection of the retinohypothalamic tract [3]. This discovery opened up a series of ablation and transplantation studies where Stephan and Zucker as well as others, determined that bilateral destruction of the SCN permanently eliminates circadian rhythmicity in drinking and locomotor activity of rats [4-7]. On the other hand, the transplantation experiments showed that just a small SCN neural graft can restore the circadian rhythms in a lesioned arrhythmic animal. Regardless of the host genotype or the direction of the transplant, the restored clock's properties were always displaying the period of the donor genotype suggesting that the circadian period must be determined by the neuronal cells of the suprachiasmatic region [8].

A diversity of neuronal cells, differences in neuropil volume and a variety of afferent/efferent connections indicate that the SCN is rather a heterogeneous than a homogenous unit. Although many electrophysiological studies and experiments on dispersed suprachiasmatic nucleus cultures have demonstrated a strong competence of SCN neurons to be an individual cell - autonomous oscillators [9, 10], it still was not clear whether or not all neurons of SCN exhibit rhythmic properties.

Anatomically, the SCN is divided into two main partitions: 'shell' (dorsomedial area) and 'core' (ventrolateral area) [11] (Figure 2). Intense research showed that cells possessing an endogenous rhythmicity in electrical activity, gene expression and protein phosphorylation are mainly localized in ventromedial, dorsomedial and rostral parts of the suprachiasmatic nucleus collectively called 'shell' (the distribution of these cells produce a shell like shape). The study of this SCN subdivision by multi - electrode approaches revealed that the cells of the dorsal as well as ventral part exhibit spontaneous oscillations that persist over several days [12]. Furthermore, the shell's molecular rhythms were confirmed by the identification of the rhythmic expression of immediately early genes such as *Fos*, *JunB* or *Fra2* in dorsomedial and rostral regions [13-16]. Vivid search for signs of rhythmicity within the SCN shell, led scientists to the discovery that protein phosphorylation, e.g. phosphorylation events occurring in mitogen–activated protein

kinase cascades (MAPK), could serve as a valuable marker for endogenously rhythmic cells [17]. For instance, Gau and colleagues found that peak levels of phosphorylated CREB (pCREB) in the mouse SCN appear during the subjective night, however, the regional localization of endogenously rhythmic pCREB was not showed [18, 19]. To the contrary, the levels of phosphorylated ERK were rhythmic with a clear peak during the mid-subjective day and immunoreactivity of ERK overlapped with the distribution of Vasopressin - positive cells of the dorsomedial and ventrolateral part of the SCN [20].

The second subdivision of the central SCN called 'core', seems to lack endogenously rhythmic cells. This part of the SCN has been characterized based upon light activated clock gene expression [21]. In contrast to the shell, which contains a large population of arginine vasopressin (AVP) and calretinin (CAR) expressing neurons, the core -- a site of retinal inputs implicated in entrainment -- comprises predominantly VIP - (vasoactive intestinal polypeptide) and GRP - (gastrin releasing peptide) positive neurons co-localized with GABA [11, 22].

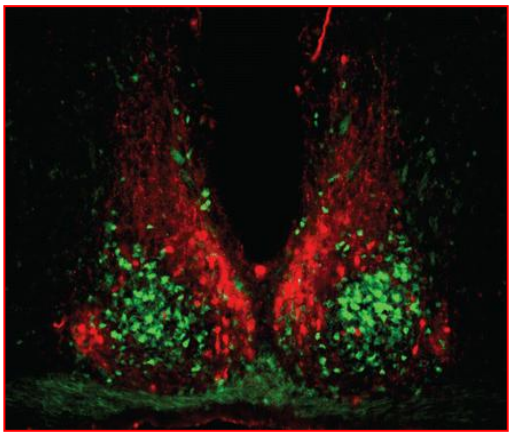


Figure 2 Coronal section of mouse SCN. Ventral core region is visualized by green fluorescent protein expressed in GRP neurons (green) and the dorsal shell region is delineated by immunofluorescent labeling for AVP (red). Optic chiasm is located below the SCN and third ventricle is between left and right SCN. Reproduced from Karatsoreos et al., 2004 [23].

Despite the structural and circadian heterogeneity of dorsal and ventral part of the SCN [24, 25], the central oscillator is able to conduct the physiological timing of the whole organism in respect to the external changes in the environment. Until now, we have quite broad knowledge about the mechanisms of external entrainment of the main oscillator (*discussed in details in the Chapter 1.2.3.*), but there is less known about the mechanism of the intra - neuronal synchronization within the SCN. Several laboratories found that neuropeptides such as vasoactive intestinal polypeptide [26], gastrin-releasing peptide, neurotransmitter γ-

aminobutyric acid [27] signals via G- protein subunit $G_{i/o}$ [28], as well as gap junctions [29], are implicated in the interneuronal synchronization and the concept of neuronal coupling based on the circadian neurotransmitter release regulation became a subject of intense study [30-33].

At the level of the SCN, the VIP neuropeptide signaling pathway is the most prominent player of inter-neuronal synchronization of firing rate and molecular pacemaking [34, 35]. VIP neuropeptide, which is rhythmically expressed by the ventrolateral neurons, acts through the G protein-coupled receptor VPAC2 (encoded by *Vipr2* gene) that activates adenylyl cyclase [36], intracellular cAMP, protein kinase A (PKA) and ultimately CREB phosphorylation. The acute induction of *Per* genes expression by phosphorylated CREB is then followed by repression of PER and CRY in the subsequent cycle and a new phase of the circadian rhythm is re-established. (See chapter 1.2.2.1 for circadian clock genes) Most of our understanding about VIP function arises from the knockout animal studies. For instance, transgenic mice lacking VIP or VPAC2 receptor expression ($Vip^{-/-}$,$Vipr2^{-/-}$) display similar behavioral deficits [34, 36, 37]. Moreover, molecular rhythms of core clock genes have been found to be strongly attenuated in $Vipr2^{-/-}$ mice [36, 38]. Along with these molecular and behavioral phenotypes, altered firing rate rhythms and disrupted synchronization have been also reported in $Vip^{-/-}$ and $Vipr2^{-/-}$ SCN [34, 39].

Apart from the VIP, GRP neurotransmitter found in the ventral SCN and its BB2 receptor expressed in the dorsal SCN seem to play a role in the circadian rhythmicity of the SCN. Several studies revealed that GRP administration evokes phase shifts in firing rate rhythms, in SCN slices and behavior, while deletion of BB2 leads to attenuation of the phase – shifting effects of GRP [40-42]. In addition, Brown et al. showed that GRP can effectively promote cellular rhythmicity in $Vipr2^{-/-}$ SCN slices [38]. So, as we can see here, the rising body of evidence suggests involvement of GRP in the SCN synchronization, however, the exact role of this neuropeptide in the neuronal coupling still remains undisclosed.

Beside the neuropeptidergic signaling, gap junctions between two adjacent neurons promote synchronization and secure precision in the circadian activity and behavioral rhythms. Gap junction channels are typically formed by a connexin 36 protein. Long et al., in 2004, showed that Cx36 knockout mice elicit dampened circadian behavioral rhythms and delayed activity onset in constant darkness. Moreover, an electrical coupling between SCN neurons was lost. Taken together, electrical synapses in the SCN help to synchronize its firing activity that is necessary for normal circadian behavior [43].

1.2.2 Molecular bases of circadian clockwork

The process that generates and maintains self-sustaining oscillations in most model organisms is thought to be approximately 24 – hour period transcriptional and translational oscillatory machinery. Extensive investigation of the genetic basis of circadian rhythmicity in many model systems such as cyanobacteria, fungi, protozoans, insects and mammals revealed the existence of core clock genes whose undisturbed action regulates many aspects of biological rhythmicity.

1.2.2.1 Core Clock Genes and their implication in circadian machinery

Core clock components are defined as genes whose protein products are essential for the generation of circadian rhythms [44]. The process of understanding the architecture of these components as an oscillating clock began with the discovery of clock mutants in the filamentous fungus *Neurospora crassa* and the fruit fly *Drosophila psuedobscura*. The identification of *Neurospora* clock mutants [45] led to the discovery and cloning of the Frequency locus (*frq*), one of the first known circadian clock genes [46]. Similarly, in *Drosophila,* the pioneering forward genetic screens of Roland Konopka and Seymour Benzer led to the discovery of the *Period* (per) locus [47, 48].

Subsequent research in both organisms showed that their circadian oscillators are based upon autoregulatory feedback loops of transcription and translation [49] (Figure 3). This basic structure has been conserved in all species studied. Interestingly, however, these loops are not essential in cyanobacterial clocks, which are based upon cyclical rhythms of phosphorylation of clock components [50]. Although cyclical post-translational modifications of clock components play a prominent role in metazoan clocks as well, current evidence suggests that transcription/translation feedback loops consisting of both positive and negative elements are essential to their primary mechanism [51].

In mammals, the positive elements of these loops include members of helix-loop-helix (bHLH)-PAS (Period –Arnt-Single-minded) transcription factor family, CLOCK and BMAL1. In some tissues including brain and liver, the NPAS2 protein can also play a pivotal role[52]. CLOCK or NPAS2 and BMAL1 heterodimerize and initiate transcription of target genes, which contain E-box *cis*- regulatory sequences. Negative feedback is achieved by a complex of other components including the PERIOD1-3 (PER) and CRYPTOCHROME1-2 (CRY) protein products. The genes encoding these products are activated by CLOCK:BMAL1 heterodimers via

E-boxes, and their cognate proteins then translocate back to the nucleus to repress their own transcription by preventing CLOCK:BMAL1 complex binding [49, 53]. The CIPC protein, which interacts with CLOCK:BMAL1 complexes and inhibits their activation activity, probably also plays a role [54].

A second type of loop is formed by CLOCK:BMAL1 heterodimers that activate the transcription of retinoic acid-related orphan nuclear receptor genes *Rev-Erbα* and *RORα* [55]. REV-ERBα and RORα subsequently compete dramatically to bind retinoic acid-related orphan receptor response elements (ROREs) which are present in the *Bmal1* promoter. RORα, as well as related proteins ROR β and γ) activate the transcription of *Bmal1* and REV-ERBα (and probably its sister protein REV-ERBβ) repress it [55, 56].

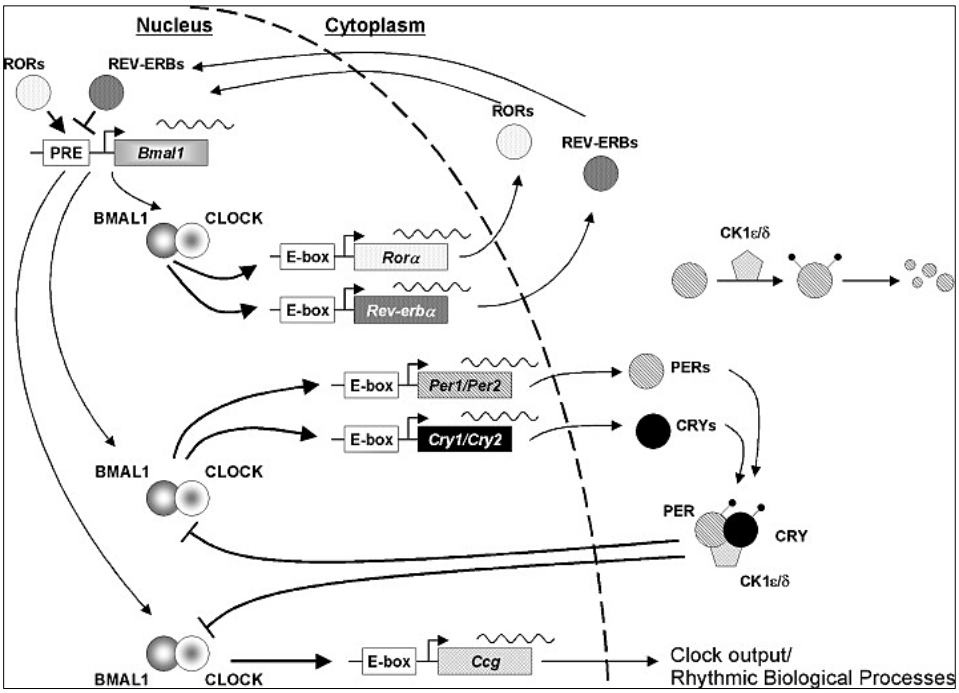


Figure 3 Core clock components of the mammalian transcriptional - translational feedback loops. The positive elements of the primary feedback loop are represented by the CLOCK and BMAL1, which heterodimerize and initiate the transcription of target genes containing E-box cis regulatory enhancer sequences including Periods (Per1, Per2, Per3) and Cryptochromes (Cry1, Cry2). Negative feedback is achieved by PER: CRY heterodimers that translocate back into the nucleus and repress their own transcription. Casein Kinase 1 epsilon and delta are critical factors that regulate the core circadian turnover in mammals. The second regulatory loop induced by CLOCK-BMAL1 heterodimers activates the transcription of retinoic acid-related orphan nuclear receptors (Rev-erbα, Rorα) that consequently compete to bind retinoic acid-related orphan receptor response elements (ROREs) in the Bmal1 promote. RORs activate transcription of Bmal1, whereas REV-ERBs repress the transcription process. Reproduced from Ko & Takahashi 2006 [57].

1.2.2.2 Post translational modification of clock components

Post-translational modifications of clock components and of other proteins play an important role in both loops. These modifications include phosphorylation and ubiquitination of clock components, chromatin modifications, and possibly even direct acetylation of some clock components by others. For example, PER proteins are phosphorylated by *casein kinase 1*ε and δ, and probably by other kinases as well, and these phosphorylations affect both the nuclear localization and degradation via ubiquitination [58]. Ubiquitin ligase coupling via the FBXL3 protein also affects degradation of other clock proteins such as CRYs [59, 60]. At the level of chromatin structure, circadian loci such as *Dbp* and *Rev-Erb*α change each day from a repressive to an active chromatin structure via histone acetylation and methylation [61].

Finally, CLOCK (the mouse circadian locomotor output cycles kaput) protein itself possesses a histone acetyltransferase activity and can acetylate BMAL1 [62]. Since the Clock can be acetylated, there must be also a mechanism of deacetylation. A recent report published in 2008 showed that *sirtuin1* ((silent mating type information regulation 2 homolog, SIRT1) which is a NAD^+ - dependent rhythmic deacethylase, directly acts with CLOCK. Moreover, the pharmacological blocking of the SIRT1 activity resulted in disturbances of the circadian rhythm as well as in the inability of BMAL1 acetylation [63]. The complexity of this system was worked out by another group which showed that SIRT1 binds to a CLOCK – BMAL1 complex in a circadian fashion and evokes a deacetylation/degradation of PER2 [64]. In addition to phosphorylation and acetylation, it was shown that mouse BMAL1 is rhythmically SUMOylated on a conserved lysine residue (K259) in vivo, in a CLOCK dependent fashion [65].

1.2.3 The afferent connections of the SCN

Entrainment or synchronization of the circadian system to external environmental cycles is mediated by highly organized afferent neuronal pathways (Figure 4) which are conserved among different mammalian species ranging from rodents to humans. Photic as well non-photic stimuli have been shown to modulate the rhythmicity of the main brain circadian pacemaker, which ultimately orchestrates the downstream timing in the physiology and behavior.

1.2.3.1 Photic entrainment for the suprachiasmatic nucleus

One of the most salient properties of the circadian system is the ability to be entrained by the external light – dark cycles. According to these cycles, SCN drives diurnal changes in behavior and physiology, and as it was demonstrated by many groups in controlled conditions (e.g. Constant light or constant dark) the period length of endogenous oscillations is remarkably close to 24 hours.

The Retinohypothalamic tract

In general, various photic and non – photic environmental timing cues ("Zeitgebers") are capable to entrain the circadian rhythmicity of the central pacemaker. Among many, light seems to be the most powerful photic zeitgeber.

In mammals, the transmission of the light information from the external environment into the internal milieu of the brain is mediated by highly organized retinohypothalamic tract (RHT) [3]. The retinohypothalamic pathway originates in the small population of photosensitive retinal ganglion cells (RGC) that produce an opsin-like protein melanopsin [66-68]. The importance of melanopsin, rods and cones for circadian photo - entrainment was demonstrated by Hattar and colleagues where a triple mouse knockout induced the loss of ability to entrain to light/dark cycles [69]. The axons of the RGCs mostly project into the key centers of circadian photo - entrainment such as SCN, the intergeniculate leaflet (IGL) and the olivary pretectal nucleus (OPM) of the brain. The selective lesion of the RHT fibers projecting directly to the SCN revealed the so called "circadian blindness" however, no loss of vision was determined [70].

The Geniculohypothalamic tract

Apart from the direct projection of retinal cells to the SCN via RHT, there also exists an indirect pathway called geniculohypothalamic tract, which arises from the neurons of the lateral geniculate complex, the intergeniculate leaflet (IGL) and a portion of the ventral lateral geniculate nucleus (vLGN) of thalamus [71]. The IGL receives the direct projection from the retina and consequent integration of photic and non-photic cues modulates the SCN rhythmicity [72]. Many laboratories showed that the lesion of IGL alters the circadian system response to constant light condition [73, 74].

The Serotogenic Tract

The third key afferent input to the SCN is represented by the serotogenic tract, innervating from the midbrain raphe [75, 76]. Many studies showed a significant role of serotonin in the regulation of the SCN circadian oscillator. Lesion experiments in the midbrain raphe nuclei responsible for the serotogenic projection, showed an alteration in the locomotor activity of the rats [77, 78]. Moreover, pharmacological manipulation of serotonin (5HT) synthesis and degradation supported the hypothesis of the serotogenic modulation of photic response of the main oscillator [79, 80].

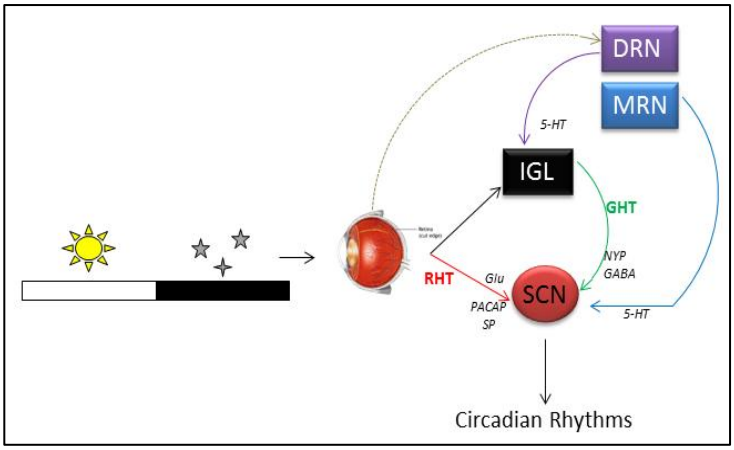


Figure 4 The most important afferent connections to the SCN. RHT: retinohypothalamic tract, Glu: glutamate, PACAP: pituitary adenylate cyclase-activating peptide, SP: substance P., IGL: intergeniculate leaflet, GHT: geniculohypothalamic tract, NPY: neuropeptide Y, GABA: γ-aminobutyric acid, 5-HT: serotonin, DRN: dorsal raphe nucleus, MRN: median raphe nucleus. Adapted from Challet E.&Pevet P.,2003 [81].

1.1.1.1.1 Signal transduction of photic messages within the mammalian SCN

The light entrainment mechanism of the core clock oscillator is mainly based on the retinohypothalamic release of the neurotransmitters from the nerve terminals. Although, excitatory amino acid glutamate was recognized as a first neurotransmitter of the RHT pathway [82], later studies showed that pituitary adenylyl cycles activating polypeptide (PACAP) is also playing an essential part in the RHT light projection into the suprachiasmatic nucleus [83-86]. Besides these two main neurotransmitters, various metabotropic glutamate receptors as well as N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors of the SCN have been found to have a crucial role in glutamatergic regulation of the circadian rhythms [87, 88]. While activation of NMDA receptors initiates phase shifts, the pharmacological blocking of these receptors prevents light induced phase shifting of the

hamster's circadian system [89, 90]. Independently from glutamate and PACAP, also other molecules have been suggested as neurotransmitters of RHT. For instance, neuropeptide Y (NPY) [91] and γ – aminobutyric acid (GABA) of the IGL innervate the rhythmicity of the clock. Moreover, L-aspartate [92], N-acetylaspartylglutamate [93], histamine [94] and the still discussed substance P (SP) [95] seems as well act as neurotransmitters involved in the light pathway.

When we take a closer look at the molecular mechanism of signal transduction pathways orchestrating photic entrainment of circadian rhythmicity (Figure 5), Calcium (Ca^{2+}) acts as a first mediator of light induced entrainment of the circadian clock. Indeed, it was demonstrated that Ca^{2+} levels regulate a variety of eukaryotic physiological processes such as cell division in the microorganism *Euglena*, leaf movement in *Trifolium reopens* or conidations in *Neurosporra crassa* [96-98]. Furthermore, Cowell reported that Ca^{2+} exhibits daily fluctuations in duration and concentration level. Circadian – like oscillations in Ca^{2+} levels persisted even when the rats were kept in constant darkness, however, this was not the case for the condition of constant light. Ultimately, the observed Ca^{2+} rhythms blocked by the application of Na^{+} or Ca^{2+} Voltage - gated channel inhibitor [99] suggest, that calcium levels could be modulated by extracellular events.

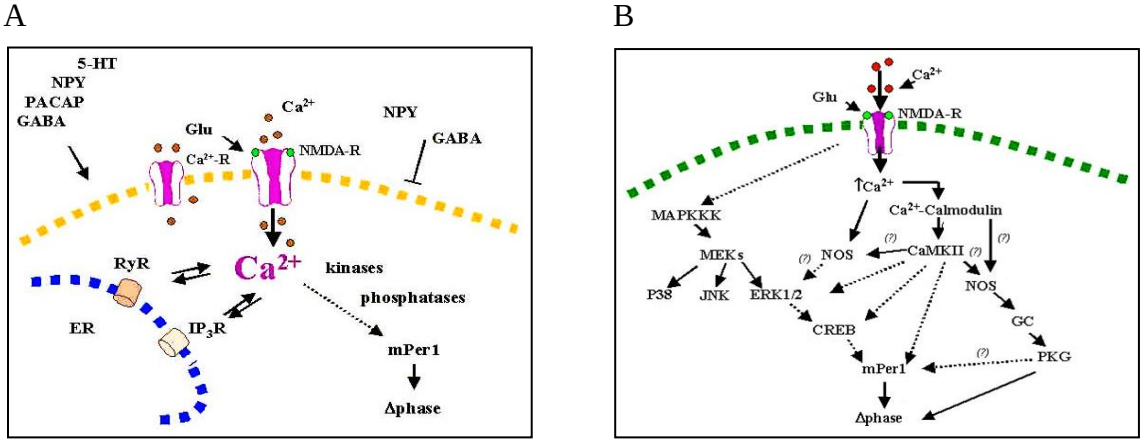


Figure 5 Signal transduction of photic messages in the mammalian suprachiasmatic nuclei. A) RHT releases glutamate onto SCN neurons and interacts with several types of receptors of which the NMDA receptor has been shown to mediate light effect. Moreover, calcium channels that are also present in the SCN could contribute to ion influx via the cytoplasmic membrane. B) Calcium influx via NMDA receptors induces CaMKinase and NOS activity that consequently modulate other kinases such as MAP kinases or PKG. Activation of the kinase cascades ultimately induce transcription factors (such as CREB) that activate clock gene transcription and clock entrainment. Question marks represent yet unknown signaling mechanisms in the SCN. Reproduced from Golombek et al.,2003 [100].

The depolarization event including immediate influx of Ca^{2+} into the retinorecipient SCN neurons appears to be mainly dependent upon a glutamate release acting on NMDA receptors [101-104]. Apart from NMDA, AMPA, Inositol triphosphate receptors (IP_3) and other metabotropic glutamate receptors, Voltage - gated Ca^{2+} channels were found to be rhythmically expressed in some immortalized cell lines [105]. Downstream of the receptors, actual Ca^{2+} activated multilevel signal transduction of the SCN relies on the harmonized interplay of various kinases (e.g. cAMP dependent protein kinase A (PKA), mitogen-activated protein kinase / extracellular signal – related kinase (MAPK/ERK), Ca^{2+}/calmodulin – dependent protein kinase (CaMK) and downstream effectors (Ca^{2+}- dependent proteases and transcription factors).

- ***cAMP/PKA/CREB/CRE signaling***

The cAMP signal transduction cascade acting via the well-known **c**AMP **r**esponse – **e**lement – **b**inding protein (CREB) is the most often studied pathway associated with the photic entrainment [18, 19, 106] and the molecular mechanism of the circadian clock [107]. CREB is a member of the basic helix-loop-helix family of transcription factors whose activation is dependent on the phosphorylation by various kinases, such as CaMK and PKA. PKA as well as the levels of cyclic 3'5'-adenosine monophosphate (cAMP), intracellular second messenger, exhibits a significant diurnal fluctuation [108]. Moreover, *in vitro* application of cAMP analogs specifically inducing cAMP signaling pathways causes a stable phase shift of the isolated circadian pacemaker [109].

Even if the relationship between the cAMP signaling pathway and circadian clock genes is still not fully understood, it is clear that core clock genes (e.g. *per1* and *per2*) [110, 111], as well as clock controlled genes containing a **c**AMP **r**esponsive **e**lement (CRE) represented by an 8-bp palindromic sequence are the targets of the cAMP/PKA/CREB/CRE pathway [112, 113].

- ***cAMP/ MAPK/CREB/CRE signaling***

Besides PKA and CREB, another downstream effector of the cAMP signal transduction cascade is critical for the clock entrainment. The highly conserved **M**itogen **a**ctivated **p**rotein **k**inase (MAPK) pathway has been found to be implicated in the regulation of many cellular processes such as cell proliferation, differentiation and cell death. In mammals, there are at least four distinct subgroups of MAPKs - extracellular signal- related kinase (ERK1/2), c-Jun N -

terminal kinase/Stress activated protein kinase (JNK/ SAPK), p38 MAP kinase and ERK5 kinase. In the context of circadian rhythmicity and photic entrainment of the clock, several studies revealed that light induced MAPK triggers the activity of Stress-activated protein Kinase 1 (MSK1) as well as p90 Ribosomal S6 Kinases (RSK) which in turn induce the phosphorylation of CREB at Ser133 and in this way initiate the regulation of CREB target genes [114, 115]. Moreover, light initiates not only the activation and the rhythmic phosphorylation of ERK [17, 116] but also JNK and p38 kinases exhibited the light induced circadian rhythmicity at the post transcriptional level [117]. Along with ERK/MAPK pathway, mTOR (mammalian target of Rapamycin) signaling revealed a 24 - hour's circadian rhythmicity which was correlated with the rhythm of the circadian clock gene *per1* at the cellular level [118]. Except the role in the photic entrainment of circadian chief oscillator, cAMP responsive MAPK signaling seems to have an essential function in the synaptic plasticity and memory consolidation. A recent study published by Eckel - Mahan and her colleagues suggested, that circadian oscillation of the hippocampal cAMP and MAPK activity could be a valuable piece of a puzzle creating the story of the persistence of contextual memory [119].

- ***CAMKII/ nNOS/NO/GC/cGK signaling***

CAMKII/nNOS/NO/GC/cGK signaling is another alternative to the SCN light activated signaling pathway. CAMKII or Ca^{2+}/calmodulin – dependent protein kinase type II is a serine/threonine protein kinase which was found to be rhythmically phosphorylated in the SCN under the entrained as well as free running conditions [120] and is assumed to have an implication in the resetting of the circadian clock by light [121, 122]. Under the entrained conditions, CAMKII is specifically phosphorylating its downstream target the neuronal nitric oxide synthase (nNOS) at Ser847. Pharmacological inhibition of CAMKII abolishes the increase of phosphorylation as well as light pulse induction of nNOS activity [120]. The main role of nitric oxide synthase is the production of nitric oxide (NO) from a precursor L-arginine and its linkage to the resetting mechanism of the clock was extensively studied [120, 123, 124].

According to the literature, the signal transduction downstream of NO could be branched into the two main divisions: The cascade which seems to be responsible for the light induced circadian phase advance and the cascade representing phase delay. The light pulse stimulation during the late night, evoking phase advance of the circadian rhythms, enhance the NO ability to

activate guanylyl cyclase (GC), which in turn elicit circadian changes in the level and activity of cGK (cyclic guanosine monophosphate dependent protein kinase, also called Protein kinase G, PKG) [125, 126]. Moreover, the pharmacological inhibition of cGK blocked light induced phase advances in vivo [127].

On the other hand, circadian phase delay seems to be induced by the activation of the rhodamine receptors, which are under circadian control [128, 129]. Despite the road junction of the NO/cGK pathways it is clear that NO and cGMP are capable to enhance a rapid and transient cGK phosphorylation of the final pathway effector transcription factor CREB *in vitro* [130], which in turn regulates transcriptional activity of the circadian clock genes.

1.2.3.2 Non-photic entrainment for the suprachiasmatic nucleus

Apart from the light orchestrating the circadian rhythmicity of the main oscillator, other non-photic cues such as induced physical activity, food or pharmacological compounds have an enormous potential to influence the rhythmicity of the circadian clock. Several studies demonstrated that time restricted access to a running wheel at a certain part of the day or exposure of the animals to a novel - wheel can successfully entrain the circadian system [131-133]. In addition, restricted access to an unlocked running wheel in sighted or anophthalamic mice also revealed the ability of clock synchronization [134] that is mediated via the geniculohypothalamic tract [135].

Equally to the non – photic arousal entrainment, circadian clock can be synchronized by the restricted food availability during the daily cycle which was characterized by modulated ‘’food anticipatory activity’’ in corticosteroid secretion, body temperature and locomotor movement [136, 137]. It was shown, that the food expectancy or food synchronization is independent from the light entrainable circadian system by several lesion studies [138-141]. Since the temple of the light entrainment is located in the rostroventricular hypothalamus, many groups tried to identify the center of the food anticipation. Until now, according to a *c-Fos* neuronal activation, all hypothalamic nuclei with the exception of the SCN and ventromedial nucleus [142], likewise reward-related areas appear to be involved in food anticipatory activity [143]. At the molecular level, food restriction is undoubtedly synchronizing peripheral clocks, however, there is no effect on the expression of SCN clock genes [144]. In contrast, rats under the light/dark cycles and timed caloric restriction showed a circadian phase advance in the locomotor activity, body

temperature and melatonin [145]. Furthermore, the timed caloric restriction was sufficient to entrain the behavioral rhythms as well as the molecular machinery of the SCN - the phase of the core clock genes has been in phase with the time of feeding [146]. In addition to these findings, restricted re-feeding of the arrhythmic rats kept under constant light, re-induced the abolished PER2 expression and behavior rhythmicity [147].

Altogether, photic and non-photic entrainment plays a decisive role in the resetting of rhythmicity of the central oscillator, indispensable for controlling many aspects of human physiology as well as behavior. In the absence of the main light zeitgeber for the SCN, restricted feeding seems to be able to entrain the SCN quite effectively. Beside basic research, understanding the entrainment mechanism for the suprachiasmatic nucleus rhythmicity could provide new insights into the clinical research of sleep and circadian rhythm disturbances and possibly improve therapeutic technologies.

1.2.4 The efferent connections of the SCN

The generation and maintenance of the circadian rhythmicity in a mammalian system originates in the suprachiasmatic nucleus of brain hypothalamus. However, other parts of the brain including thalamic nuclei, amygdala, olfactory bulbs or cerebellum [148] as well as peripheral tissues possess circadian oscillations in the gene expression [149, 150].

In general, the SCN outputs are mostly mediated by neuronal efferences, but research in the last couple of years revealed also the existence of humoral signals. A very elegant experiment done by Silver and her colleagues showed, that the transplantation of encapsulated SCN tissues into a SCN ablated animal can restore the circadian locomotor activity, however, it fails to restore many rhythms in the neuroendocrine function. The neuroendocrine reactivation requires intact SCN neural projections. The coupled action of direct neuronal projection and diffusible factors seem to be essential for conduction of the timing signals into the other parts of the brain [151]. To understand the orchestrating role of the SCN, it is necessary to know how and via which neurotransmitter system are neuronal cells able to direct their efferent projections to transmit the timing information. SCN is composed of many different transmitters expressing neurons from which arginine vasopressin (AVP, [152]), vasoactive intestinal peptide (VIP, [153]) and gastrin releasing peptide cells (GRP, [154]) represent the majority. Besides them, other neurochemical molecules such as somatostatin, calretinin, calbicistin, neurotensin, neuropeptide Y or TNFα [155] were found to contribute to the timing system [156]. Last but not least, neurotransmitters that usually act at the synapse levels such as GABA and glutamate have also been found to play an important role in the transmission of the circadian information [157, 158]. In the focus of neuroanatomy of the SCN outputs, the axonal connection mostly terminates within the SCN or along the midline of the hypothalamus, specifically paraventricular nucleus (PVT) and dorsomedial hypothalamus (DMH). Some efferent projections have been found to terminate in other hypothalamic and thalamic areas as the subparaventricular zone, the preoptic area, the bed nucleus of the stria terminals and the lateral septum [159].

Beside the direct neuronal SCN outputs, the chase for molecular connection for circadian rhythms revealed the discovery of hormonal or so called diffusible factors that also exhibited the circadian makeup of the regulation. Testosterone, thyroid hormone, corticosteroids, growth hormone, as well as melatonin [160-164], showed the circadian rhythmicity in their secretion.

1.3 Central clocks vs. peripheral oscillators

Genome - wide transcriptome analysis revealed that almost 10% of all detected genes possess rhythmic expression [165, 166]. This result actually only confirmed the fact that the circadian clocks are ticking not only in the SCN but in other peripheral cells and tissues as well [149, 167, 168]. Moreover, the molecular basis of these peripheral oscillators seems to be the same as the one orchestrating in the SCN [169]. In contrary to the SCN, circadian rhythms of most peripheral tissues seem to damp out in the absence of the SCN [170]. A closer look at the single cell resolution of cultured fibroblasts, however, revealed that the dampening of the rhythmicity is rather a question of inter - cellular desynchrony than loss of individual cellular rhythms [171, 172]. Moreover, Yoo et al. have shown that even apparently low amplitude of Per2 luciferase expression in some tissues like the liver and lungs can persist over twenty daily cycles [168].

In spite of the similarities given between the SCN and peripheral clock, scientists very often refer to a ‘’master’’ and ‘’slave oscillators’’ of the circadian system. In fact, SCN is a master oscillator, which through various signals coordinates not only the rhythmicity but also the synchronization of peripheral tissues. For instance, it was showed that the circadian phases of peripheral clocks in the SCN lesioned animals show significant differences. Moreover, Guo et al. showed that the transplantation of the SCN into the SCN ablated animal restored the phase continuity of circadian gene expression in liver, kidney, spleen, heart, skeletal muscle and adrenal medulla [173]. In addition to the SCN regulated peripheral slaves, local SCN independent oscillators in the retina and olfactory bulb can oscillate persistently without the SCN [174, 175].

1.3.1 Synchronization of peripheral oscillators

One way in which the SCN probably influences circadian physiology and gene expression is via the pituitary-adrenocortical axis, specifically via glucocorticoid hormones. Glucocorticoids have many important functions, including regulation of glucose, fat, and protein metabolism. They also have anti-inflammatory actions, and can affect mood and cognitive functions. Glucocorticoids can bind the glucocorticoid receptor (GR) a nuclear hormone receptor found in many cell types but not in the SCN [176]. It has been shown that dexamethasone, a glucocorticoid analog, can induce *Per1* expression in RAT1 fibroblasts, as well as change the

phase of circadian gene expression in peripheral tissues, but not SCN. It is clear that glucocorticoids are not the sole entraining signal from the SCN because mice lacking GR in liver possess normal circadian rhythmicity in this organ [177].

Another dominant "zeitgeber" or timing cue for peripheral circadian clocks is food itself. It was shown that the expression profile of many circadian genes in the liver and other peripheral tissues is influenced by the timing of food intake. Specifically, restricted feeding uncouples peripheral circadian gene expression from that in the suprachiasmatic nucleus [178, 179]. The speed and the degree to which an organ changes its circadian rhythm to match the timing of food uptake vary among different organs. Interestingly, however, this phase shift happens more quickly in adrenalectomized animals or in tissues lacking the glucocorticoid receptor when feeding time and photoperiod are placed in opposition [178]. Therefore, it is likely that the twin signals of feeding time and glucocorticoid secretion act separately *in vivo* to set clock phase. The exact nature of the food-induced signal is unclear, but the observation that glucose alone can phase shift circadian gene expression in cultured cells *in vitro* suggests that basic food metabolites could suffice [180]. Once again, it is unlikely that food entrainment is the only timing signal *in vivo*, because mice that are fed frequent isocaloric meals still display robust circadian rhythmicity in peripheral organs [181].

A third basic class of signal that may entrain peripheral oscillators is fluctuation in body temperature. In heterotherms such as *Drosophila* [182] and *Neurospora* [183], it has been known for some time that shallow 24-hour temperature fluctuations – e.g. 12 hours at 37°C followed by 12 hours at 33°C – can synchronize and phase-shift circadian oscillations in behavior and gene expression. More recently, however, we have shown that rhythmic body temperature can sustain peripheral circadian oscillators, and that inversion of temperature cycles in liver or brain cortex can invert circadian gene expression in these organs without affecting the phase of the SCN [184]. In spite of these promising results, temperature is also not the sole source of peripheral circadian entrainment *in vivo*, because "scrambling" of temperature cycles does not result in a loss or dampening of peripheral circadian rhythmicity.

Finally, Okamura and colleagues showed that communication between SCN and peripheral tissues can occur via a fourth channel: the sympathetic nervous system. In their study, periodically injected adrenaline in SCN-lesioned mice induced strong *Per2* expression in liver. This result suggested that adrenergic regulation influences circadian clock gene oscillations in

peripheral tissues. Moreover, electrical stimulation of the sympathetic nerve increased *mPer1* transcription mainly in the liver of these mice. As a confirmation that the sympathetic nerve is critical for sustaining *Per* gene expression in peripheral organs, the mice were injected by 6-hydroxydopamine.HCl which causes the destruction of sympathetic nerves. The expression of the *Per2* gene significantly reduced. Together, these studies demonstrated that sympathetic nerve activity plays a crucial role in the delivery of the central clock information to at least some peripheral tissues [185].

Of course, although I have evoked four distinct channels of communication in the above discussion, significant overlap is possible and even likely. For example, corticosterone release from the adrenal gland can occur via the sympathetic nervous system, and food metabolites can be brain signaling molecules. Overall, the key message is that the SCN conveys its timing information to peripheral circadian clocks through a redundant mix of direct hormonal and nervous signals, and indirect environmental ones such as feeding and body temperature that are influenced by rest-activity cycles (Figure 6).

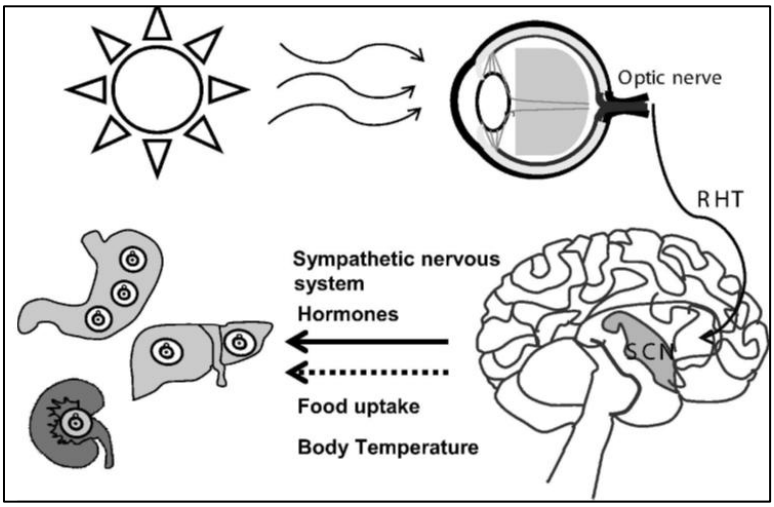


Figure 6 Organization of central and peripheral oscillators. Light is the principal timing cue that synchronizes the neurons located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, whereas the SCN communicates with peripheral oscillators via a redundant web of direct and indirect signals. For the circadian system, light is received by both rod and cone cells containing traditional opsin pigments and retinal ganglion cells containing melanopsin. This message is sent to the SCN via the optic nerve and the retinohypothalamic tract. From the SCN, a variety of signals is transmitted to peripheral oscillators. These include direct signals from the sympathetic nervous system, hormonal cues, and indirect signals such as food metabolites and body temperature that are relayed via the control of the SCN upon appetite and locomotor activity. Reproduced from Cuninkova & Brown, 2008 [186].

1.4 Measurement of Human Circadian Clocks

1.4.1 Circadian Parameters

The robust circadian oscillations are a fascinating property of various biological processes that occur within many cells and tissues. These rhythms repeat once a day, persist in the absence of any external zeitgeber and can be entrained or reset to match the local time. The nature of the circadian oscillations is thought to be determined genetically by a set of core clock genes and can be very well described by the three main variables: circadian period length, circadian phase and circadian amplitude (Figure 7).

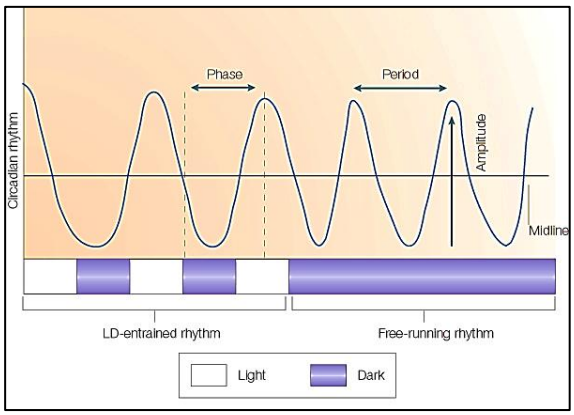


Figure 7 The basic properties of the circadian rhythms. Period - peak to peak interval of the activity, Phase - the endogenous timing of peaks and troughs of the oscillation, Amplitude - the level of expression measured from the midline to the peak. Reproduced from Bell-Pedersen et al., 2005 [187].

1.4.1.1 Period Length

Circadian clocks are able to oscillate with a wide variety of periodicities. Under constant conditions, this period has a length which is very close to 24 hours. Several studies of circadian rhythms have shown that genetically determined circadian period length could be dramatically altered by the mutations of the core clock genes. For instance, Ralph and Menaker determined that the circadian period length of Syrian hamsters carrying the *tau* (an allele of the gene *casein kinase I epsilon*) mutation exhibits shorter (20-22hrs) than normal circadian period [188]. In addition, Vitaterna et al. showed that the *Clock* mutants display an abnormally long circadian period, which eventually leads to a loss of circadian rhythmicity in constant dark conditions [189].

1.4.1.2 Circadian Phase

Circadian clock has an ability to synchronize to the external time via external environmental cues. These zeitgebers dictate the endogenous timing of peaks and troughs collectively called a relative circadian phase. In human studies, several markers like melatonin, cortisol or even temperature (Chapter 1.4.1.) could be used to determine the circadian phase of an endogenous pacemaker. Although SCN neurons are competent self-sustained oscillators able to oscillate for more than 50 days [170] most of the peripheral oscillations damp out within a few cycles. This declining pattern was further investigated and Yoo et al. showed that the dumping differences between SCN and peripheral tissues seems to be a matter of quantitative rather than qualitative properties of peripheral oscillators. Moreover, he showed that peripheral tissues are capable of persistent rhythmicity for up to 20 days independently of the SCN input[168].

1.4.1.3 Amplitude

Along with the phase, light also affects the amplitude of circadian rhythms. The measured amplitude of a circadian rhythm represents the half distance from the maximum to the minimum of the obtained circadian rhythm. Several studies revealed that while the light reduces rhythm's amplitude to nearly zero during so called "critical phase" in the mid-subjective night when phase shifts are maximal, controversially amplitude increases by light pulses during the subjective day when phase shifts are minimal [190-193]. In human studies, both phase and the amplitude of melatonin, core body temperature or even cellular gene expression can be used to describe the properties of the circadian system.

1.4.2 Human circadian markers

Circadian oscillator of the central nervous system regulates the physiology (hormone excretion, blood pressure, temperature) and behavior (mood, sleep, alertness etc.) within the 24-hours cycles synchronized to the external environment. Since this oscillator cannot be studied directly in humans, various circadian markers such as melatonin, core body temperature and cortisol have been used to reveal the function of human clock giving core oscillator.

1.4.2.1 Melatonin

Melatonin, N-acetyl-5-methoxytryptamine, is a hormone of the pineal gland whose circadian secretion is regulated by the SCN. Multiple enzymatically regulated synthesis of melatonin from tryptophan includes a rate limiting step catalyzed by serotonin-N-acetyltransferase (AA-NAAT) whose activity rhythm underlies the rhythmic formation of melatonin [194]. At the molecular level, the entire circadian secretion of the mammalian melatonin begins by the adrenergic innervation of the pineal gland followed by the binding of the norepinephrine to a β_1 – adrenergic receptor and activation of the cyclic AMP (cAMP) - signaling pathway [195], which modulates the levels and activity of AA-NAT via transcriptional and posttranslational mechanisms (Figure 8Figure 8). This control mechanism involves a harmonized interaction of distinct transcription factors. At the first place, increased levels of cAMP mobilize cAMP - dependent protein kinases (such as PKA) which in turn activate CRE – binding protein (CREB). Phosphorylated CREB binds to the cAMP responsive element of AA-NAT promoter region and activate transcriptional activity of this enzyme [196-198]. Beside the CREB based induction, nocturnally elevated levels of norepinephrine also provokes a rise of the mRNA of an inhibitory acting transcription factor called inducible cAMP early repressor (ICER) - an induced isoform of cAMP-responsive element modulator (CREM) [199]. In the rat pineal gland before the dawn, ICER causes a rapid decline of increased AA-NAT mRNA levels what in turn decrease the production of melatonin [200]. A recent *in vitro* study showed that phosphorylated CREB accumulates at the beginning and declines with the rising amount of ICER during the second half of the night. This switch ratio between phosphorylated CREB and ICER is responsible for the generation of *in vivo* dynamics in mRNA as well as protein level of AA-NAT. Moreover, silencing of the ICER expression in pinealocytes enhanced the production of melatonin [201].

Mammalian SCN expresses at least two (MT1 and MT2) high affinity G – protein coupled melatonin receptors via which melatonin affects many mammalian biological functions (sleep – wake cycles, circadian rhythms etc.) [202]. Beside MT1 and MT2, the third melatonin binding site *MT3* (quionone reductase 2) has been identified in the Syrian hamster hypothalamus [203], however, its function is still poorly understood [204].

Many different studies using mammalian cell lines (e.g. CHO, NIH 3T3 or HEK) expressing the recombinant forms of MT1 and MT2 revealed the linkage of multiple G - protein induced

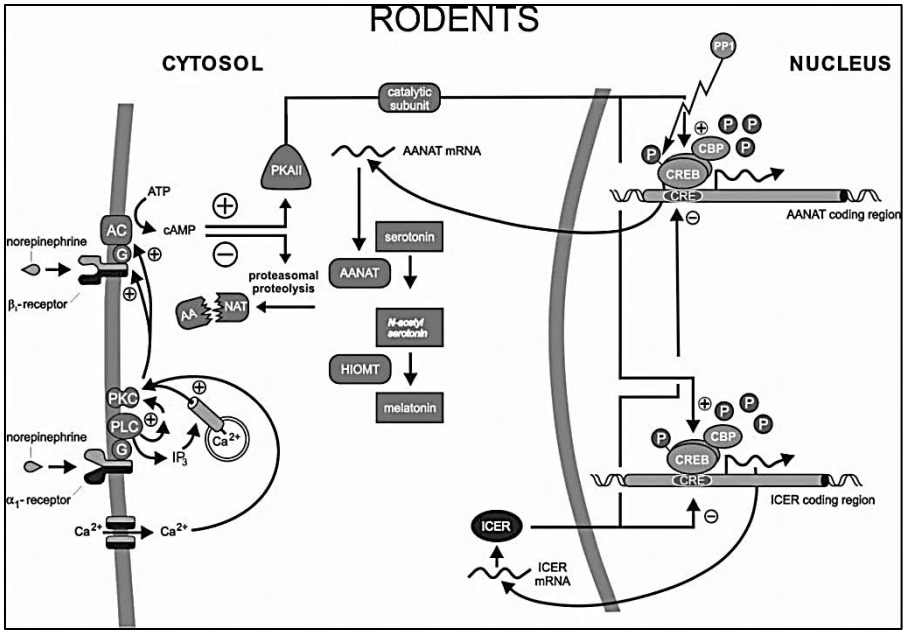


Figure 8 Regulation of melatonin synthesis in rodents. AANAT-arylalkylamine N-acetyltransferase; AC - adenylate cyclase; AMP - adenosine monophosphate; ATP - adenosine trisphosphate; CBP - CREB binding protein; CRE - cyclic AMP response element; CREB - CRE binding protein; G - GTP-binding protein; HIOMT - hydroxyindole O-methyltransferase; ICER – inducible cyclic AMP early repressor; IP3 - inositoltrisphosphate; P - phosphate groups; PKAII – cyclic AMP-dependent protein kinase type II; PKC - protein kinase C; PLC - phospholipase C. Adapted from Ackermann & Stehle, 2006 [205]

signal transduction cascades to the activation of these receptors. On one hand, activation of MT1 melatonin receptors via G_i proteins (such as G_{i2} and $_{Gi3}$) inhibits forskolin activated formation of cAMP, which in turn down-regulate PKA activity to phosphorylate cAMP responsive element binding protein (CREB) [206, 207].

On the other hand, MT1 activation increases phosphorylation of MEK1/2 (extracellular signal – regulated kinase kinase 1 and 2) and ERK1/2 (extracellular signal –regulated kinase 1 and 2) [208]. Moreover, activation through G_q protein has been associated with the elevation of phosphatidylinositol turnover and calcium mobilization [209, 210] and it was shown that the release of G protein subunit β and γ potentiate stimulation of phospholipase C mediated by prostaglandin F_{2a} and ATP [211]. Furthermore, activation of MT1melatonin receptors was found in the regulation of ion channels - activation of G protein coupled inwardly rectifying potassium channel (GIRK) Kir3 via MT1 melatonin receptor increase the conductivity of potassium channel [212]. Last but not least, Chan and colleagues showed that activation of MT1 melatonin receptors stimulated c-Jun N-terminal kinase activity through G_i and G_s, G_z and G_{16} proteins [213].

Beside MT1 receptors, the activation of MT2 melatonin receptors also leads to an inhibition of cAMP production induced by forskolin as well as modulation of c-Jun N-Terminal kinases via Gi and G16 proteins [213]. In spite of these similarities, activation of MT2 but not MT1 melatonin receptors can modulate cGMP levels through the soluble guanylyl cyclase pathway, in a dose dependent fashion [214]. In addition, activation of the MT2 melatonin receptors inhibits $GABA_A$ receptor in the hippocampus [215] and increase PKC activity with the circadian phase shift of the neuronal firing activity in the SCN of rat [216].

Autoradiography with 2 – [^{125}I] iodomelatonin binding, Western blot, immunohistochemistry analysis or RT-PCR have been used to determine the distribution of MT1 and MT2 melatonin receptors in different areas of the central nervous system and peripheral tissues. Starting at the circadian system, the mammalian retina expresses both MT1 and MT2 melatonin receptors which are prominent in regulation of a rod phototransduction pathway and in the maintenance of the circadian variation of an intraocular pressure [217-220].

Downstream of the retina, mRNA and proteins for MT1 and MT2 melatonin receptors were found in the mammalian SCN. Activation of these receptors in the SCN evokes several cellular responses. For instance, in SCN slices melatonin receptors activation inhibits neuronal firing [221] and function as an inducer of the phase advances at subjective dusk and subjective dawn [222] acting via MT2 receptors whose activation modulate the activity of PKC. Furthermore, von Gall et al. showed that in the mouse SCN melatonin inhibited PACAP - regulated CREB phosphorylation mediated via MT1 melatonin receptors activation [223].

Moving along the Hypothalamic – Pituitary – Gonadal Axis, where hypothalamus produces a gonadotropin-releasing hormone, pituitary gland producing luteinizing and follicle –stimulating hormone and gonads producing estrogen and testosterone, activation of melatonin receptors seems to play a role in the reproductive system and seasonality as well [224-226].

Another target of melatonin action via MT1 receptors includes modulation of glucose homeostasis and energy balance through the direct effect on adipocytes and pancreas cells [227-229]. Additionally, melatonin was linked to a regulation of cardiovascular [230] and immune system [231]. Since melatonin has a direct effect on cell proliferation and cytokine secretion, it has been suggested as oncostatic agent [232, 233]. According to melatonin mediated responses, melatonin receptors seem to be challenging targets in the development of the new treatment for sleep, circadian, metabolic, endocrine disorders and cancer.

Since it is not possible to directly measure the functional timing of the SCN endogenous clock, melatonin rhythms determining the clock output are usually used to assess circadian phase position. Melatonin levels can be easily measured in human blood or saliva but the detection of melatonin metabolite 6-sulfatoxymelatonin from urine also showed a strong correlation with plasma and serum levels of melatonin [234]. Circulating levels of melatonin are very often preferred as a circadian marker due to the robustness in the presence of various external factors. For example, while excessive intake of carbohydrate causes significant changes in core body temperature, concentration of melatonin remains practically unaffected [235]. However, numerous researches showed that ocular light is causing an acute suppression of nocturnal plasma melatonin that is dependent on various light parameters (such as intensity, duration, wavelength and time of administration) [236] and other factors such as individual susceptibility or experimental design. It has been found that melatonin used as a circadian phase marker should be measured as a dim – light melatonin onset, since it's thought to be minimally masked by exogenous factors. Beside the light, melatonin can be influenced by posture [237], physical exercise [238], caffeine and sleep [239] likewise by the distinct groups of drugs such as benzodiazepines and β- blockers [240, 241].

1.4.2.2 Core body temperature

The first description of the circadian rhythm in core body temperature (CBT) was described by Gierse in 1842. This rhythm is characterized by a nocturnal decrease and daily maximal increase of the body temperature. Aschoff and his colleagues showed that the rhythms in core body temperature are caused by changes in heat production and heat loss [242]. The nadir of the core body temperature was traditionally used to assess the circadian phase of the endogenous oscillator [243, 244]. Likewise the stable melatonin marker, CBT rhythm is also ''masked'' and influenced by many different factors such as sleep, activity level or posture [245-247] as well as by external conditions like ambient temperature, sound or bright light [248, 249]. To minimize these effects, many constant routine protocols have been implemented [243, 250].

1.4.2.3 Cortisol

Since the endogenous pacemaker generates the circadian rhythms in hypothalamic-pituitary-adrenal axis through a multisynaptic suprachiasmatic adrenal pathway, the secretion of cortisol is

highly rhythmic [251]. In contrast to melatonin, the peak levels of cortisol have been determined in the early morning with forthcoming decline during the daytime and a nadir at midnight. Genetic twin studies determined that the timing of this cortisol nadir is a robust marker of human circadian phase and is dependent on the endogenous period length [252]. Serum free cortisol diffuses into saliva and the measurement of salivary cortisol reflects the concentration of serum free cortisol more accurately than measurement of total cortisol in the serum [253]. Beside the cortisol nadir, the onset of the evening rise or the end of the quiescent period are the most often analyzed parameters of cortisol [254, 255]. Although cortisol seems to be a reliable marker to define the endogenous circadian phase, there are several factors influencing cortisol secretion. Among many, light was shown to significantly increase morning – cortisol peak [256, 257]. In addition, physical stress, aging and sleep – wake cycles have also been shown to play role in the secretory activity of cortisol [254, 258, 259].

1.4.2.4 Transcriptomics and Metabolomics

As it was already discussed in previous chapters, circadian rhythms exist in a variety of biological processes and control a broad spectrum of physiology such as sleep/wake cycle, hormone and neurotransmitter excretion. It is also well known that altered circadian cycle might lead to metabolism defects that are characterized for example by obesity or other metabolic syndromes [260, 261]. The main regulator of metabolism has been found in the liver. Several studies revealed that the liver transcriptome as well as proteome oscillates in expression activity that is extensively dependent on the food zeitgeber [262-264]. Beside the liver, very recent study revealed that approximately 15% of plasma and saliva metabolites are also under the circadian control [265]. Moreover, other biochemical molecules such as cAMP or NAD^+ [107, 266, 267] as well as several blood metabolites also revealed circadian oscillations that are dependent on an intact endogenous clock [268]. Taken together, all discussed results suggest that various so called small molecule biomarkers that are under the control of the circadian clock might provide a valuable approach for the determination of human circadian properties in the future, such as circadian phase as well as they can serve as a tool to detect human pathologies.

1.4.3 In vivo protocols to study human circadian properties

The measurement of human circadian properties such as free running period, circadian phase or circadian amplitude required an implementation of various protocols, which eliminate all influencing external factors, the so called "masking factors" and can desynchronize internal timing from the external.

1.4.3.1 Phase Response Curve, Free running period

One of the principal difficulties in determining the genetic linkage between human behavioral disorders and the circadian oscillator is simply measuring the properties of the human circadian clocks that determine the behavior. In principle, two different properties can be measured: free-running period, or the length of one oscillation under constant environmental conditions, and phase response / entrainment, the ability of the clock to alter its phase in response to external stimuli. Though formally distinct, these two properties are under normal circumstances inter-related because the mechanism by which circadian clocks are synchronized by light is non-parametric [182]. In other words, to entrain to the daily light-dark cycle, the circadian oscillator responds differently to light at different phases of its cycle. This differential effect is most easily visualized as a Phase Response Curve (PRC), which plots phase shifts of a circadian rhythm as a function of the circadian phase that a stimulus, or zietgeber, is given. The characteristic form of this curve was first described by DeCoursey thirty years ago in the flying squirrel [269], and can be determined by a number of different protocols, as described by Aschoff (1965) [270]. From such a curve, one can make deductions about the phase, period and amplitude of the central oscillator (Figure 9).

In human beings, the measurement of either free-running period or phase response is extremely expensive and labor-intensive because it demands extensive subject observation under controlled laboratory conditions. Nevertheless, reliable estimates have been made by a variety of methods for both human period length (24.2-24.4 hours) [271, 272] and the human phase response to bright light pulses [273]. By comparing human free-running period length to behavioral chronotype, it has also been possible to observe a correlation between these properties [274, 275]. Similar observations of morning-type behavior in individuals of short endogenous period and evening-type behavior in individuals of long endogenous period have been observed previously in other animal systems [276].

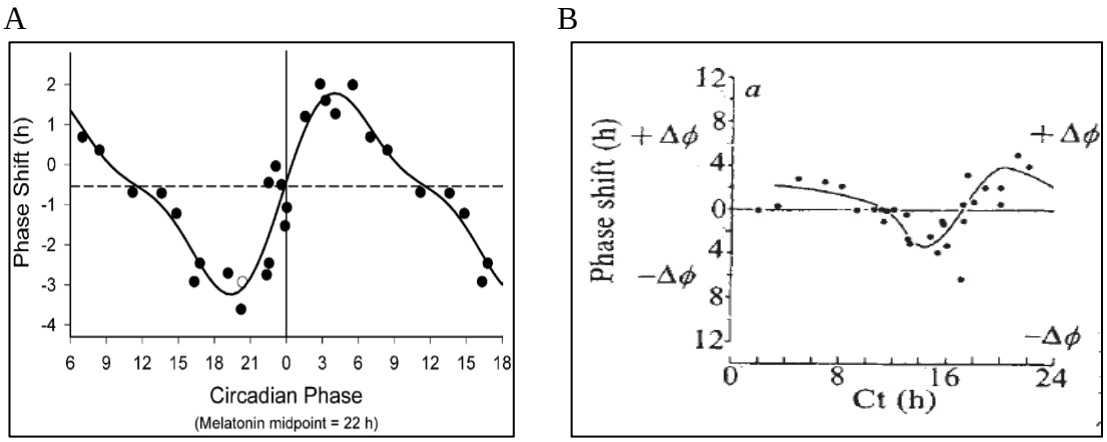


Figure 9 Phase response curves in different organisms. A.) The phase response curve to a bright light stimulus in humans. Circadian phase was measured as the midpoint of melatonin expression. (Panel reproduced from Khalsa et al.,2003 [277].) Positive values reflect phase advances and negative values reflect phase delays. B.) Phase response curve to a bright light stimulus in the cockroach Nauphoeta cinerea. Circadian phase was estimated via the timing of locomotor activity. (Panel reproduced from Saunders & Thomson, 1977 [278])

PRCs can also be performed with other phase-shifting agents such as drugs or temperature. For example, a physiological dose of the hormone melatonin shifts circadian rhythms in humans according to a phase-response curve (PRC) that is nearly opposite in phase with the PRCs for light exposure: melatonin delays circadian rhythms when administered in the morning and advances them when administered in the afternoon or early evening. This difference points to multiple different pathways for the entrainment of the human circadian oscillator. More practically, the human melatonin PRC also provides critical information for using melatonin to treat circadian phase sleep and mood disorders [279].

1.4.3.2 Forced Desynchrony

The first forced desynchrony protocol done in 1938 was performed by Nathaniel Kleitman who scheduled subjects to live on artificial daylights in Mammoth Cave in Kentucky. In these conditions (e.g. Sleep-wake cycles scheduled into the extremely short 20 - hours or long 28 - hours day lengths), the circadian clock cannot entrain and starts to oscillate with its own endogenous period [280]. Many scientists followed Kleitman and began to study the circadian clock using the same principle. In 1995 Dijk et al., scheduled eight men subjects to a 28-hours sleep/wake cycle for 36 days what resulted in sleep episodes at all phases of the endogenous circadian cycle [281]. Since this protocol was too elaborate and time consuming, Hiddinga used 120 hours lasting protocol where the circadian variation in the body temperature was studied [282].

1.4.3.3 Constant routine protocol

Beside the forced desynchromy, constant routine protocols have been used to reveal unmasked circadian rhythms. In this protocol, subjects under the time - free conditions are usually kept in constant posture, under the dim light and controlled temperature. Moreover they are given small isocaloric snacks as well as water each 2 hours during the 40 hours of total sleep deprivation [283]. By this way, protocol minimizes masking and can provide reliable estimates about the phase or amplitude of the circadian rhythm by measuring core body temperature or melatonin [284]. Since these protocols are still demanding the subjects, several modification such as "constant bed rest" protocol [285] and "multiple nap" protocol has been developed [286]. These protocols are preventing an accumulation of sleep pressure, so an esteemed masking factor is reduced and the circadian rhythm appears very clearly.

Taken together, there are various possibilities to measure *in vivo* circadian rhythm in humans. Biological markers such as melatonin, core body temperature and cortisol levels have been used to determine the phase of the circadian rhythms and if these parameters are measured longer than for a one cycle also the endogenous period of the circadian pacemaker could be determined.

1.4.4 *Ex vivo* and *In vitro* studies of human circadian properties

Since the human *in vivo* studies are quite expensive and labor demanding, the big challenge was to develop methods that would be effective and robust in defining inter-individual phenotypic differences but still sufficiently cost-effective to screen a large number of individuals. A duplication of the circadian oscillators in the periphery gives an enormous advantage to determine the various circadian properties by using tissue explant as well as cultured cells. Brown and his colleagues developed a cell culture method which allowed them to measure inter-individual variation in circadian rhythms directly in human skin biopsy fibroblasts. By introducing a stably integrated lentiviral – luciferase circadian reporter, these authors showed large inter-individual differences in the circadian period length. The same reporter system was employed in mouse circadian mutants and showed the correlation of the circadian period length from mouse fibroblast cultures with behavioral rhythms of these clock mutants [287]. Moreover, it was showed that there are individual differences in circadian phase-shifting responses and amplitude in fibroblasts from human subjects, and these differences are correlated with their diurnal phase preferences [288]. Later on, Pagani et al., also measured circadian period in human

primary fibroblasts and for the first time showed the positive correlation between circadian period length in physiology and in the gene expression of fibroblasts measured via lentivirally delivered circadian reporter [289]. Beside "human *in vitro*" bioluminescence measurement, luciferase reporters found an application in many different circadian studies starting from the simplest microorganisms to more complex mammalian species.

1.4.4.1 Peripheral Oscillators as tools to study human behavior

Although the central clock of the SCN that specifies behavior is quite difficult to access at a molecular level, the circadian clocks that exist in peripheral cells appear to use many of the same components. Hence, a major breakthrough for mammalian circadian biologists has been the ability to use these cells as proxies -- albeit imperfect ones -- for the clocks of the SCN. The period of electrical firing in the SCN has been observed to correlate closely with the period of behavior in hamsters [290], so it is possible that there exists a direct parallel between human circadian behavior and the molecular properties of human peripheral oscillators. Importantly, genetic differences appear to manifest themselves in both peripheral and central oscillators [291, 292]. Though the clocks of peripheral and central oscillators are similar, they are not identical. In mice containing a PER2: luc fusion protein, the free-running period of luciferase expression varies by up to three hours in explants from various tissues. Fibroblast period was one of the closest to that of the SCN [293]. Nevertheless, genetic differences appear to be exaggerated in fibroblast circadian period compared to that of the SCN. For example, the disruption of the *Per1* gene in mice results in a one-hour shortening of the behavioral circadian period in the mouse, but a four-hour reduction in the period of fibroblast gene expression [291, 294]. Similar exaggerations can be seen for multiple other circadian mutations [291, 294]. Recent research suggests that this increased robustness of the central clock versus peripheral clocks is due to intercellular coupling of neurons within the SCN. This coupling can occur via both neuropeptidergic mechanisms and electrical synapses [295, 296]. Another source of difference between SCN and peripheral oscillators may occur because of their use of slightly different suites of the circadian clock proteins. For example, the CLOCK protein appears to be essential to proper clock function in peripheral oscillators, but dispensable for SCN oscillations and circadian behavior *in vivo* in mice [52]. Similarly, the tau mutation in syrian hamsters that result in shortening of the free-running period of behavior also affects the SCN and peripheral tissues

differently [297]. Thus, the correlation between the circadian period of behavior or SCN electrical firing and the circadian period of peripheral gene expression is not exact. Indeed, neither period is an exact value. Different free-running periods of behavior can be measured for human beings kept under conditions of forced desynchrony (a day: night cycle so long or short that the endogenous circadian clock "free-runs" to reflect its endogenous period rather than adjusting to the environment) and under constant conditions [298, 299]. Similarly, the period of circadian gene expression in fibroblasts can be altered by changing growth conditions such as incubation temperature and media supplements like serum. [276].Importantly, however, these properties appear to be trait-like: under similar conditions, they remain constant for a given individual.

Our laboratory has been able to measure the period length of circadian gene expression in fibroblasts by using lentiviruses containing a circadian reporter (Figure 10) [287]. Populations of cells from different individuals measured in this fashion showed an average period that corresponded to what has been measured for human behavior in other studies, but a standard deviation that was much broader among different individuals, implying the same sort of peripheral cell "exaggerations" to which we have alluded earlier [294]. It is possible that this enhancement of individual differences makes fibroblast period a good choice for a quantitative trait in genetic mapping studies to find the genes responsible for differences in human circadian behavior. Such differences are likely to arise from a variety of different underlying genetic causes. A recent study of fibroblast circadian clocks in human morning-types and evening-types showed not only period differences among cell cultures from some individuals in the two groups, but also differences in the amplitude of circadian gene expression and in the phase-response properties of cells from people with opposite behaviors but identical free-running fibroblast periods [276]. Specifically, it was possible to determine phase-response curves subject fibroblasts to forskolin, a chemical stimulus that activates adenyl cyclase via a mechanism reminiscent of the actions of the photopigment melanopsin upon the circadian oscillator. These curves clearly show that the phase of circadian gene expression can be altered by factors other than endogenous period length. Indeed, it has been shown previously that the reduction of circadian amplitude in mice containing a mutant CLOCK allele can enhance phase-shifting by light in these mice [300]. Results using human peripheral fibroblasts imply that human circadian behavior may be determined by a rich mixture of causes including the period length, amplitude,

and phase-resetting properties of the endogenous circadian oscillator, and that these properties can be studied in peripheral fibroblasts.

It is possible that fibroblasts or other peripheral cell types might be used not only in the mapping of the genetic variations responsible for differences in human daily behavior, but also for the diagnosis of underlying causes of human circadian disorders in some individuals. For example, Vanselow and colleagues introduced a mutation in PER2 believed to be responsible for human Familial Advanced-Phase Sleep Syndrome (FASPS) into fibroblasts, and were able to recapitulate the phase-advance in the behavior of FASPS patients as an advanced phase of clock gene transcription in synchronized FASPS fibroblasts. Subsequent molecular analyses allowed them to show effects of this mutation upon phosphorylation at multiple sites in the PER2 protein, and to further demonstrate that these modifications affected both PER2 protein stability and nuclear localization [301].

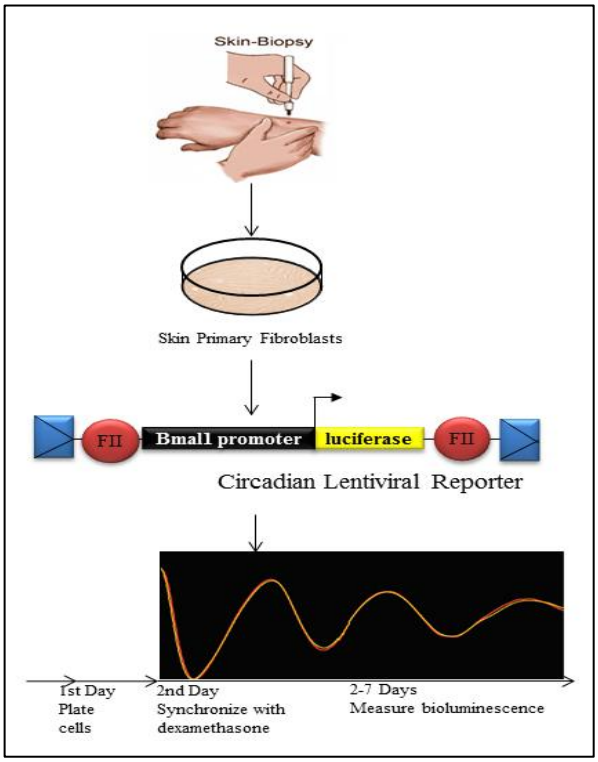


Figure 10 Measurement of the circadian period length in human skin fibroblasts. Primary skin fibroblasts are derived from the simple punch biopsy. Transduction of such primary fibroblasts with the circadian lentiviral reporter allows for the direct real-time measurement of human circadian period in primary cells.

1.5 Circadian Rhythm in Physiology and Human behavior

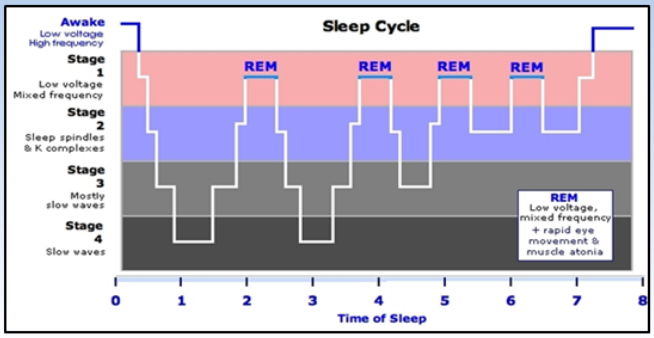
The circadian system has an important influence on human physiology and behavior. Indeed, considering the extent of circadian regulation, it is perhaps not surprising that disruption of biological clocks has a negative effect. One of the most obvious manifestations is jetlag, misadjustment of circadian phase due to travel. Links have also been established between circadian irregularities and psychiatric disorders, including various forms of depression and mania. Prolonged disruption of circadian rhythms is believed to have significant adverse health consequences on peripheral organs outside the brain as well, particularly in the development or exacerbation of cardiovascular disease and cancer [302, 303]. Conversely, chronopharmacology – the timing of treatment in coordination with the body clock – may significantly increase efficacy of various therapies, and reduce drug toxicity or adverse reactions [304].

1.5.1 Human chronotype

Even under normal conditions, the complex nature of circadian behavior is evident from the fact that phasing of the cycle during the day varies widely for individuals, resulting in extremes colloquially called larks and owls. Morningness-eveningness, or "chronotype", is an individual characteristic that refers mostly to the phase of sleep timing [305]. Because of this effect of the circadian system upon sleep, most circadian rhythm disorders *(discussed in the chapter 1.5.3.)* are therefore classified as sleep disorders. Nevertheless, circadian sleep disorders and true sleep disorders are likely to be mechanistically unrelated, and therefore it is both scientifically and clinically relevant to distinguish between them.

Sleep is an active and as yet poorly understood process during which many physiological and cerebral events occur. Indeed, even sleep itself is actually an ultradian process represented by the alternation of different electrophysiologically defined sleep states (Box 1). In general, the daily sleep-wake cycle is under circadian control, although the urge to sleep appears to be controlled by brain functions that are independent of the circadian system [306]. This independence led Borbely and colleagues to propose a model for the regulation of sleep that includes a homeostatic process that accumulates during wakefulness and diminishes during sleep, as well as an independent circadian drive [307]. Each of these processes can operate independently; thus, sleep duration is not correlated with sleep phase in humans [308].

Box1 : Sleep stages. Sleep is represented by four main stages; Stage 1 that is very light sleep. During the stage 2 the brain activity change slightly and peaks of waves become higher and slightly more random. K-complexes that are drastic bursts of activity can occurred. These are followed usually by sleep spindles. In the stage 3 and 4 brainwaves are much higher and less frequent. In stage 3 approximately 25% of brainwaves are delta waves which are followed by about 50% delta and 50% theta in stage 4. Before entering REM sleep, the stages reverse. Adapted from Hartmann, The function of sleep, 2006



1.5.2 Clock genes and sleep

A number of studies suggest that the clock genes contribute to the control of sleep homeostasis [309]. Starting with the positive elements of molecular circadian clockwork, homozygous *Bmal1*$^{-/-}$ mice exhibiting arrhythmic circadian phenotype [310] showed longer REM and NREM periods under LD and DD condition and delta power during the light phase seemed to be constantly elevated. On the other hand, under the condition of sleep deprivation, *Bmal1* mutants revealed an attenuated homeostatic response [311]. Besides *Bmal1*, a second positive element of transcriptional/translational loops of the circadian machinery, namely a *Clock* gene, has also been found to be implicated in sleep homeostasis. *Clock* mutation prolonging circadian period, caused around 2h reduction of a total time of sleep under the light dark conditions and had 51% smaller increases in REM sleep during 24h recovery than wild-type mice [312]. Furthermore, studies focused on a functional paralog of *Clock* gene, *Npas2*, demonstrated that *Npas2* deficient mice showed approximately 25% decrease in NREM and REM sleep [313] and under sleep deprivation these mutants exhibit a smaller compensatory increase in NREM sleep and in delta activity [314].

In addition to the positive players, the impact of negative loop elements representing *Cry1/2* and *Per1/2* has been also the focus of several studies directed on sleep homeostasis. While single *Cry1* and *Cry2* knockouts did not show any significant effect on sleep homeostasis, both NREM and EEG delta power were increased during the light phase in *Cry 1/2* $^{-/-}$ mice. Moreover, these mice showed attenuated responses to prolonged wakefulness [315]. Similarly, single *Per* gene

mutations exhibited an altered 24h distribution of sleep but normal responses to sleep deprivation [316, 317]. However, arrhythmic *Per1/2* double knockout animals showed decreased NREM and REM sleep periods during the light phase and slightly increased delta power after sleep deprivation [318]. Although many laboratories investigated the role of the core clock genes in sleep homeostasis, *Dbp* knockout mice were used as first animal model to challenge this task. *Dbp* – deficient mice showed reduced REM sleep during the light phase, likewise a decreased delta power activity in the dark. In addition, under the sleep restriction protocol, significantly attenuated REM responses were observed [319].

The last but not least, a fascinating study was done recently by He and coworkers. They identified a miss-sense mutation in the human *DEC2* gene responsible for the sleep length in humans. When this mutation was reproduced in mice, the decrease of the total sleep including NREM and REM phases was obtained without effect on the circadian period [320].

To conclude, clock genes do not only influence sleep architecture, but they might also be implicated in sleep function of living organisms. In many cases, clock gene mutations and sleep disorders share the same phenotypes and a better understanding of the underlying mechanisms can help to improve the therapeutic strategies of many associated disorders.

1.5.3 Circadian rhythms sleep disorders and their treatment

The so-called "circadian rhythm sleep disorders" can result from alterations in the properties of the endogenous circadian clock (for example delayed sleep phase and advanced sleep phase) or changes in the physical environment in relation to the endogenous clock (shift work disorders and jet leg). In the former class, which is unrelated to human choice, genetic variations in circadian genes have been found to associate with these disorders. Table 1 shows a list of polymorphisms that have been linked to known clock genes.

Disease	Location of SNP	Affected genes
Familial advanced sleep phase syndrome (FASPS)[321]	662 S/G 44 T/A	*Per2* *Ck1δ*
Extreme diurnal preference[322]	T2434C	*Per1*
Bipolar disorder (BD)[323, 324]	11p15 1p36.23 12q12-q13	*Bmal 1* *Per3* *TIMELESS*
Delayed sleep phase syndrome (DSPS) [325]	647 V/G S408N	*Per3* *CK1ε*
Seasonal affective disorder (SAD)[326]	471 L/S	NPAS2
Non-Hodgkin's syndrome (NHS) [327]	394 A/T	*NPAS2*
Schizophrenia[328]	31111 T/C	*Clock*
Winter depression[329]	SNP 10870 SNP rs2290035	*Per2* *Arnt1*

Table 1 Reproduced from Cuninkova & Brown, 2008 [186]

1.5.3.1 Familial Advanced Sleep Phase Syndrome

One of the most-studied syndromes is the Familial Advanced Sleep Phase Syndrome (FASPS). Individuals with this syndrome can wake up and go to sleep hours earlier than normal. This phase change is believed to be related to a change in the endogenous free-running period of the human circadian oscillator. Normally around 24 hours, it has been measured to be only 20 hours in an individual from an extensively-studied FASPS lineage [330]. In this lineage, the

source of the circadian change has been mapped to a change from serine to glycine at residue 662 of the *Per2* gene. This mutation abolishes one of the phosphorylation target sites for CK1ε. *In vitro* experiments confirm that the mutation reduces the ability of CK1ε to phosphorylate PER2 protein [331]. A second independent lineage confirmed the importance of casein kinase-mediated phosphorylation to human chronotype. This time, the corresponding mutation was mapped to a missense (T44A) change in the CK1δlocus that results in a lower kinase activity *in vitro* and shorter periods when introduced into mice *in vivo* [321].

Treatment: Chronotherapy, timed light exposure and pharmacotherapy with melatonin include the therapeutic approaches for Advanced Sleep Phase Syndrome. Delay of the circadian rhythms timing by bright light therapy improved sleep and daytime performance in older individuals suffering by ASPS [332, 333]. In addition to light treatment, different administration strategies for melatonin medication were a topic of broad investigation. Based on the phase response curve to melatonin, early morning application of melatonin would fall in the curve's advance portion and therefore cause advance of the sleep-wake cycle timing, however many questions have been raised considering the safety about taking melatonin – a sleep promoting compound, in the morning [334, 335].

1.5.3.2 Delayed Sleep Phase Syndrome (DSPS)

Not surprisingly at all, not only extreme early phase but also extreme late phase has been correlated with genetic alterations in clock genes. For example, a genetic association study of 105 individuals has linked a length polymorphism in the *Per3* gene to Delayed Sleep Phase Syndrome [336]. Other studies have seen various degrees of association not only between DSPS and this *Per3* allele [337], but also other *Per3* alleles [338] and a *CLOCK* allele [339], though not all studies have drawn the same conclusions [340]. In addition, an allele of CKIε has been found to be anticorrelated with DSPS, implying that it may play a protective role against this syndrome. Although the basis of altered chronotype in this suite of mutations is unknown, many of them are presumed to alter endogenous period length in fashions similar to FASPS, principally because there is an association between free-running period length and entrained behavioral phase in humans and in other animals [341]. Moreover, genetic mapping studies in inbred strains of laboratory mice suggest that many different loci other than those of known clock genes might influence free-running behavioral period [342].

Treatment: Effective treatment for circadian rhythms disorders requires a combined approach based upon re-alignment of the circadian clock with desired sleep and wake schedule. Correct sleep hygiene, avoidance of bright light in the evening or increasing light exposure in the morning could be basic steps in the treatment of DSPS. For instance, bright light of 2500lux for 2h in the morning has shown to successfully induce phase advance of the circadian rhythm of core body temperature in DSPS patients [343, 344]. Beside the light therapy, pharmacological administration of melatonin has shown promising effects on DSPS [345].

1.5.3.3 Non-24-h sleep-wake syndrome (FRD)

Non-24-h sleep-wake syndrome (free-running disorder, FRD) occurs when endogenous circadian pacemaker is not able to entrain to a 24h period and its start to "free-run" usually longer than 24h. Most individuals with this syndrome are totally blind and the failure to synchronize circadian rhythms is coupled to the lack of photic input to the circadian pacemaker [346, 347]. This incapability to entrain causes a chronic pattern of a disease with 1-2h daily delay in sleep onset and wake time. Beside the studies in blind individuals, few reports showed the presence of non-entrained type of sleep disorder in sighted individuals [348], however the etiology of this disorder is unknown.

Treatment: Both behavioral and pharmacological options are available for the treatment of free-running disorder, depending on whether the patient is blind or sighted. For blind people timed exposure to non-photic entraining agents and melatonin administration are the primary therapies. An appropriate melatonin timing and dosage are the factors limiting the success of the treatment and several studies showed that melatonin should be administrated a few hours before the predicted endogenous melatonin onset (DMLO). Patients after this scheduled therapy showed less wake after sleep onset and better sleep efficiency, however, discontinuation of this treatment causes the recurrence of the free-running rhythm [349-351]. Due to the rareness of the FRD in sighted individuals, most published reports represent case reports. The basic approach of the treatment includes exposure to bright light during the day as well as maintaining a regular sleep, wake and work scheduled as well as exogenous melatonin application [348].

1.5.3.4 Irregular sleep wake rhythm (ISWR)

Very poor sleep consolidation where sleep and wake periods are distributed in three or more short bouts throughout the 24h is the main feature of Irregular sleep-wake disorder (ISWR). The disrupted entrainment pathway to the SCN results in a decreased exposure to environmental synchronizing agents. Although the prevalence of this disorder is unknown, the individuals suffering by this disease also exhibit neurological disorders such as mental retardation, brain injury or dementia [352, 353].

Treatment: The final targets in the treatment of ISWR are to increase the duration of consolidated sleep periods during the night and improve daytime function. A multi-therapeutic approach coupling bright light exposure and behavioral modifications seem to be effective for young and older patients with ISWR [354-356].

1.5.3.5 Jet leg

Temporary misalignment among the endogenous circadian sleep-wake rhythm and the external environment due to a change in time zones characterize a transient Jet leg disorder. Usual Jet Leg symptoms include insomnia, daytime sleepiness as well as decreased performance [357] and few studies showed a rising severity of these symptoms in elderly individuals[358, 359]. In addition to *in vivo* studies, the disruptive effects of jet leg have been also demonstrated at the molecular level of the SCN clock genes as well as in the clock machinery of peripheral tissues [360, 361]. Beside these studies, researches also looked at the pathological consequences of Jet Leg and showed that the disruption of the circadian clock coordination might have a pivotal role in the malignant progression[362].

Treatment: Treatments for jet lag are mainly directed on adjustment of the endogenous circadian cock to the destination time zone. Non-pharmacological approaches as proper sleep hygiene, adjusting the sleep schedule prior to travels or timed light exposure have been shown to help to decrease symptoms and accelerate circadian adjustment [363, 364].

1.5.3.6 Shift work disorder (SWD)

Night work and shift work has been associated with negative effects such as shortened and disturbed sleep, decreased alertness cognitive decline and risks to cardiovascular and gastrointestinal health [365, 366]. Moreover, a number of health problems like metabolic

syndrome, ulcer disease, cancer pathologies and mental and physical fatigue have been reported [367-369].

Treatment: Similar to jet lag, appropriately timed light therapy and maintained entrainment to the shift schedule reduces the symptoms of shift work disorder. Several light therapy directed studies used intensities of the light between 1,200 and 10,000 lux for a period of 3 to 6 hours during night shifts. In addition to the circadian phase re-setting effects of the light, melatonin treatment of SWD has been a focus of several studies, however several mixed conclusions have been raised [370-373].

1.5.4 Circadian Mood disorders

One striking feature of circadian rhythm sleep disorders is that they are often associated with other mood disorders. Indeed, a part of this association is by definition: an established clinical symptom of diseases like major depressive disorder (MDD) and bipolar disorder (BD) is abnormal sleep/wake, appetite and social rhythms [374, 375], which are also the hallmarks of circadian rhythm disorders. Nevertheless, an increasing body of evidence suggests that there exists a compelling genetic basis for this correlation. In bipolar patients, a single nucleotide polymorphism in the 3'flanking region of the *CLOCK* gene associates with a higher recurrence rate of bipolar episodes [376]. This mutation is specific to bipolar depression: a similar association was not found in the Major Depressive Disorder (MDD, or unipolar depression) [377]. Another mutation, this time linked to the onset of illness in Bipolar Disorder, has been localized to the glycogen synthase kinase 3β promoter [378]. This enzyme is the target of lithium, a common treatment for BD, and can phosphorylate the clock component REV ERBα [379]. It is likely that multiple other genetic associations remain to be found between the various forms of depression and clock genes. A pilot study of circadian genes and their linkage to Bipolar Disorder 1 unearthed *Bmal1, Timeless*, and *Period3* as possible candidates [380].

Schizophrenia is also accompanied by severe sleep-wake disturbances, and has been associated with clock gene polymorphisms in this and other preliminary studies [381]. Finally, dementia has also been linked with circadian dysfunction in Huntington's Disease, though in this case the dysfunction is believed to be a neurological consequence of HD pathology upon the SCN, rather than a genetic linkage between dementia and circadian rhythm disorders [382].

Treatment: Light therapy, melatonin administration as well as pharmacological strategies by using antidepressant drugs and mood stabilizers are the possible choices for therapy. However better understanding the principles and the molecular basis of circadian rhythm disorders are necessary steps to correctly phenotype the disease and accurately set up the medical treatment.

1.6 Interplay of the circadian signaling pathway with other major signal transduction events

Many organisms possess internal highly organized systems that allow them to orchestrate their physiology and behavior to better adapt to our constantly changing environment. At the most basic level, complex mechanisms exist side by side to help cells to deal with environmental adaptation and anticipation. When change occurs in a predictable way such as cyclic changes of day and night, an internal circadian timing system anticipates these changes and regulate daily rhythms of target genes expression necessary to respond to these events [383]. On the other hand, unanticipated changes (e.g. exposure to the damaging UV light) trigger the activation of non-circadian cellular responses such as conserved MAPKinase and other major signaling pathways at any time of the day [384, 385] (Figure 11).

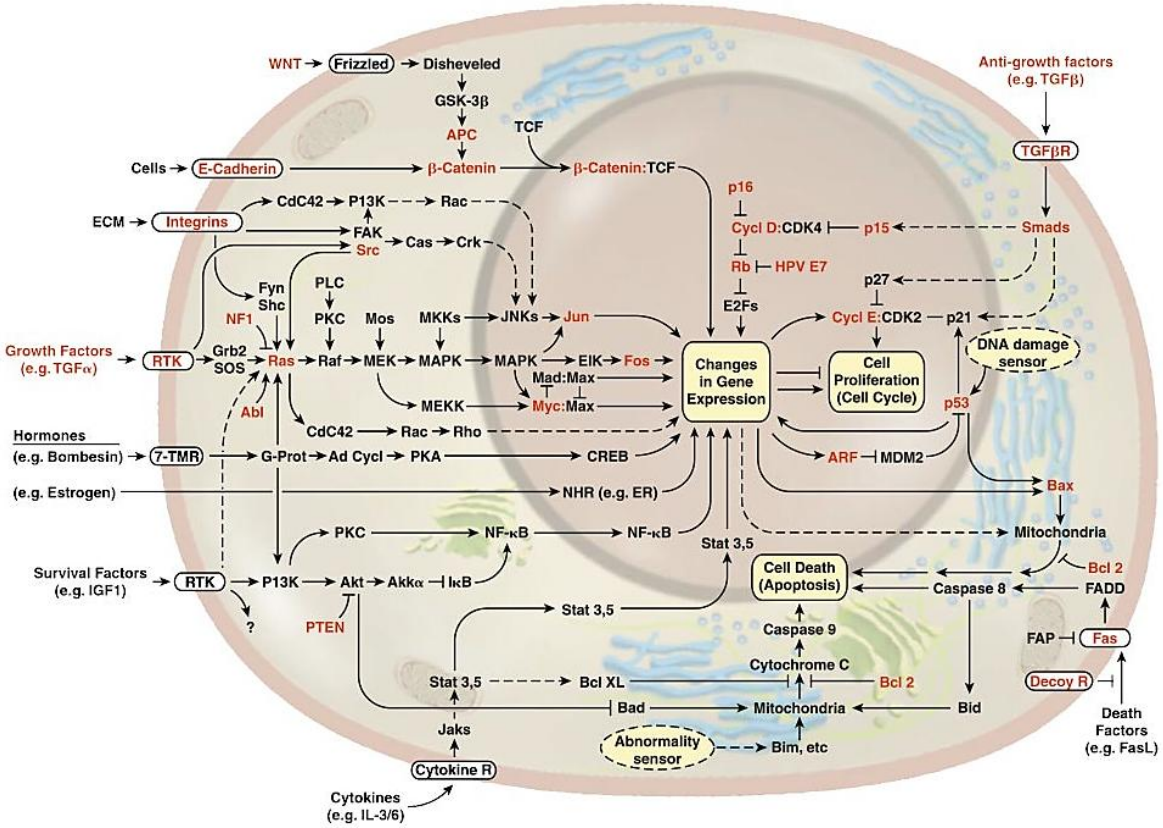


Figure 11 Complex circuitry of signal transduction pathways. The genes highlighted in red have been found to be implicated in the epigenetic reprograming of the signaling circuit in cancer cells. Reproduced from Hanahan et al., 2000 [386].

1.6.1 Circadian clock and Mitogen-activated protein kinase pathways

MAPK pathways mediate information routes in which cells can transduce environmental signals in the inside of the cells to conduct appropriate responses [387]. Three consecutively activated protein kinases - MAPKKK, MAPKK and MAPK compose the module of mitogen-activated protein kinase signaling. These components are often used in multiple pathways resulting in gene expression, cell division, proliferation and cell survival [388, 389]. Four distinct MAPK family subgroups have been identified in mammals: 1) Extracellular signal-regulated kinases (ERK), 2) Stress-activated MAPKs (SAPKs) and ERK/big MAP kinase, 3) c-Jun N-terminal kinase (JNKs) and 4) the p38 family of MAPKs [390]. Although circadian and MAPK response mechanisms have been thought to be independent, increasing evidence indicates that they share some remarkable linkages [391]. The association of MAPK and the circadian clock has been shown in several studies in insects, birds or mammals [17, 392, 393]. For instance, rhythms in the phosphorylated form of ERK were obtained in the mouse SCN under constant environmental conditions with the peak expression occurring during the day [17]. Moreover, daily rhythms in phospho -38 and JNK of low amplitude have been reported [117, 393].

1.6.2 Circadian clock and immune signaling pathways

Multiple mechanisms also conduct efficient and rapid inflammatory responses including feed-forward and amplification loops. About a decade ago, NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells), JNK (c-Jun N terminal kinase), p38 MAP kinase, STAT (signal transducers and activators of transcription) and P13K (phosphatidylinositol-3-kinase) pathways have been identified as interconnected, cross talking signaling cascades implicated in immune activity. Circadian signaling pathways as one of the cross talking pathways is also linked to immune responses and time of day-dependent variation of the immune system parameters such as lymphocyte proliferation, activity of natural killer cells, humoral immune responses likewise levels of cytokine and serum cortisol has been reported [394-398]. Moreover, it was shown that circadian signaling pathway modulates inflammatory gene expression. Keller et al showed that approximately 8% of the macrophage transcriptome is under circadian control. This circadian transcriptional regulation was found at four levels of LPS-induced immune response: 1) Factors regulating LPS binging to TLR4 (Toll-like receptor 4), 2) components of MAPK pathway (ERK)

and cytokine protein processing (MEK1), 3) NF-κB and AP-1 (activating protein-1) transcription factors involved in pro-inflammatory cytokine transcription and 4) components controlling cytokine mRNA stability and localization [399]. Broadening our knowledge in circadian immune regulation will have a strong impact on understanding of pathological inflammatory responses and certainly might help to improve the development of anti-inflammatory compounds.

1.6.3 Circadian clock, cell cycle and cancer

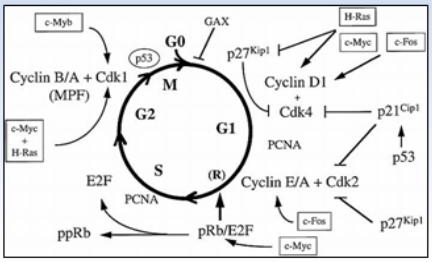
In this thesis, I developed a novel cell – based system that allows us to study human inter individual variation in the circadian clock properties as well as in other vital signaling pathways (Chapter 3.2) and their relationship to disease, especially cancer (Chapter 3.3). Therefore, the current chapter 1.6.3 devoted to the circadian clock, cell cycle and cancer is followed by the discussion about signaling pathways implicated in the cancer pathogenesis.

Several molecular and conceptual similarities are shared by the circadian clock and the cell cycle. (General scheme and description of the cell cycle is in the Box 2) Clock and the cell cycle, periodically repeated within 24 h cycles are intrinsic to most cells and interlocked autoregulatory loops rely on sequential phases of transcription, translation, post-translational modification and degradation [400]. The circadian clockwork regulates the cell cycle related gene expression and can oscillate accurately and independently [401]. At the molecular level, circadian control of key cell cycle gene regulators, such as *Wee1* (GM2/M transition), *c-Myc* (G0/G1 transition) and *Cyclin D1* (CCND1, G1/S transition) has been reported in mammals.

Wee1 is a nuclear kinase belonging to the Ser/Thr family of protein kinases that inactivate the cell division cycle 2 (CDC2) by phosphorylation at T15 to control the G2/M transition. The binding of CLOCK/BMAL1 to the E-boxes of *Wee1* promoter region activates a robust circadian oscillation of this gene in the liver as well as a deregulated expression of *Wee1* was demonstrated in *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ arrhythmic mice [401, 402]. Under normal conditions, CLOCK-BMAL1 heterodimers bind to the E-boxes of the promoter region to inhibit the transcription of *c-Myc* gene. Except the cell proliferation, *c-Myc* transcription has been found to play a crucial role in apoptosis. Oncogenic transformation mediated *by c-Myc* must overcome the proapoptotic activity where modulation of p53 regulated apoptosis play an important role [403]. Overexpression of this gene can induce DNA damage and compromise p53 function, most possibly via a mechanism mediated by reactive oxygen species (ROS) [404, 405]. The last key

gene in the cell cycle which is under the circadian clock control is *Cyclin D1*. Overexpression studies of this gene showed its implication in tumorigenesis as well as in breast cancer pathology with poor diagnosis [406].

Box 2: Cell cycle. G0 quiescent cells enter G1 – phase of the cell cycle, in which cell grow in size in response to mitogenic signals, such as growth factors. During G1- phase, activated protein kinases (CDKs ; *cyclin dependent kinases* that bind to a protein partner, a *cyclin*) send a signal that cell division process has begun. The first group of cyclins that are activated in this stage of the cell cycle are cyclins D. In the late G1 – phase cell are committed to entering the next phase of the cell cycle called the restriction point (R). Activation of the E – type cyclins along with a CDK catalytic partner is a characteristic feature of R. After the R, a cell begins to replicate its genetic material in the S – phase. The replication machinery of cells depend on the protein kinase activity of cyclin A coupled to CDK1 and CDK2. At the completion of S – phase, cells enter the G2 – phase during which CDK1 replaces CDK2 and couples with either cyclin A or cyclin B to catalyse the phosphorylation of proteins specific to the G2 and M phases of the cell cycle. Mitosis is the stage of the cell cycle in which cell physically separates into two daughter cells. B – types cyclins remain active during M-phase, but their activity decline once cell division is complete and the two daughter cells once again enter G1-phase. Reproduced from Braun-Dullaeus R.C. et al, 1998.



Taken together, the normal regulation of signal transduction events among various cell types is evidently a crucial checkpoint of many functions of living organisms. But what happens when the functions of these signal transduction pathways, including circadian signaling become altered or deregulated? Accumulating epidemiological and genetic evidence indicate that disruption of specialized pathways might be directly linked to the cancer pathogenesis (Figure 12). For instance, an altered circadian signaling pathway controlling the cell proliferation can result in a transformation and cancer [407, 408]. Modified light schedules disrupt the circadian timing mechanism in rodents resulting in a significant increase of the frequency of tumors [409]. As well as transgenic mice with defective circadian rhythms exhibited an accelerated malignant growth [410], also mice deficient in *mPer1* or *mPer2* clock component have an increased incidence of tumors, where the tumors have lower *hPer1* and/or *hPer2* levels [411-413]. Several human studies also contributed to support an association between defects in circadian rhythms and cancer development. For instance, the expression of all three *Per* genes has been found deregulated in breast cancer cells and mutations in NPAS2 have been linked with an increased risk of breast cancer and non – Hodgkin's lymphoma [411, 414]

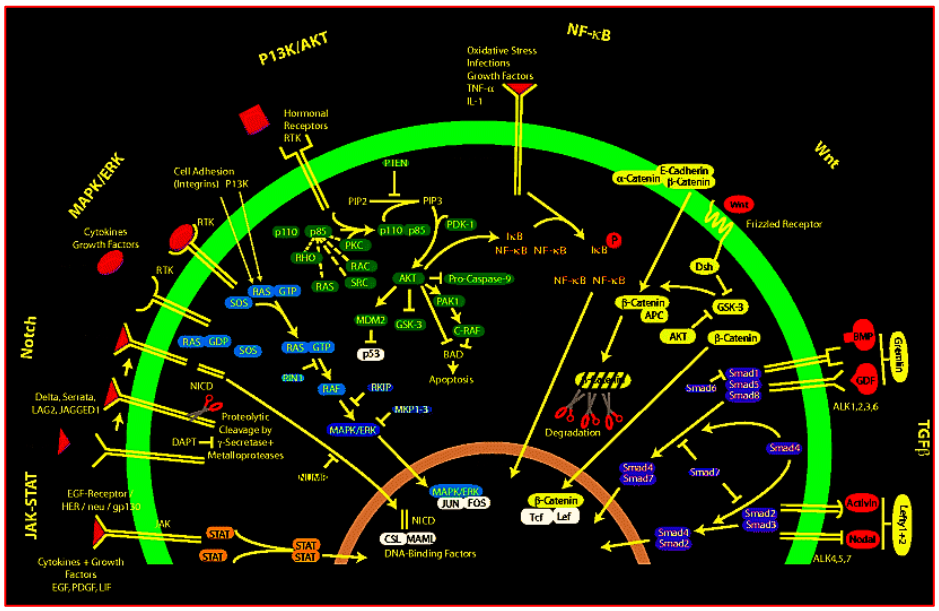


Figure 12 The most common signaling pathways altered in the cancer. Adapted from Dreesen et al., 2007 [415].

1.6.3.1 JAK/STAT signaling pathway and cancer

Apart from the direct link among the circadian signaling dysfunction and tumorigenesis, many other conserved signal transduction pathways regulating cell growth and differentiation are almost invariably altered in cancer. For example, the JAK/STAT signaling pathway contributes to tumorigenesis. These receptor-associated Janus Kinases (JAKS) phosphorylate tyrosine residues of the ligand bound receptors. Phosphorylated interacting Signal Transducers and Activators of Transcription (STATs) form homodimers that shuttle to the nucleus and regulate the transcription of a wide variety of genes[416]. The JAK/STAT pathway has an important role in mediating apoptosis, cell differentiation and proliferation in a response to growth factors and cytokines. Mutations as well as the amplification of various compounds of this pathway such as HER2 (Human Epidermal Growth Factor Receptor 2), epidermal growth factor-receptor have been found in mammary, breast, brain and stomach tumors [417-419]. In addition, constitutively activated STAT3 is a key marker of lung, breast, head/neck and hematologic tumors [420].

1.6.3.2 Notch signaling pathway and cancer

One of the hematologic tumors represents T-cell acute lymphoblastic leukemia that is caused by unrestrained proliferation of immature T-cells. Mutation in the Notch signaling pathway has been associated with almost 60% of human T- cell acute lymphoblastic leukemia. Beside human

lymphoma, recent studies showed an implication of abnormal Notch signaling in breast tumors, melanoma, medulloblastoma and ovarian cancer. The possible mechanism of cancer initiation via Notch signaling includes activation of its downstream target *c-myc* whose deregulation has been found in a wide range of cancers [421-423].

1.6.3.3 MAPK signaling pathway and cancer

The third pathway found to be deregulated in a broad spectrum of human tumors is the SOS – Ras – Raf – MAPK signaling cascade. The RAS-Mitogen activated Protein kinase signaling plays a crucial part in cell adhesion, proliferation, migration and survival [424]. RAS, a small membrane bound GTPase is activated by Son Of Sevenless (SOS) - a guanine exchange factor. Upon receptor activation, activated GTP-bound RAS activate RAF kinase that consequently induces mitogen-activated protein kinases (MAPK/ERK). ERK1/2 translocate to the nucleus where they induce the *Jun/Fos* transcription factors. Many mutations of this transduction cascade occur in RAS and RAF resulting in constitutive activation of this pathway. While colon and pancreatic cancer's hyper-proliferative states are linked to RAS mutations, two thirds of all melanoma are the result of mutations in RAF [424, 425].

1.6.3.4 PI3K/AKT signaling pathway and cancer

Similarly to MAPK/ERK pathway, the phosphatidylinositol 3-kinase / protein kinase B (PI3K/AKT) pathway responds to a variety of extra- and intracellular stimuli via harmonized action of hormonal receptors, transmembrane tyrosine kinase-linked receptors and intracellular factors that regulate cellular proliferation, cell death and cytoskeletal rearrangements [426, 427]. The basic elements of this biochemical network include growth factors activated receptor tyrosine kinases (RTKs), which then induce two key signal transduction components: the small GTPse Ras and the lipid kinase PI3K. Alterations in the PI3K pathway are common in cancer and play a role in neoplastic transformation [386]. Mutational activation of PI3K has been found in many different cancers such as breast, bowel and ovarian cancers, head and neck and cervical squamous cancers, gastric and lung cancers as well as in glioblastoma multiform and medulloblastomas [428-431]. Most of these tumors harbor activating mutations on the master regulators of this pathways (K-ras, H-ras, N-ras, the p110α PI(3)K subunit and RTKs) or inactivating mutations in negative regulators of these proteins (phosphatase and tensin

homologue (PTEN) and neurofibromin 1 (NF1)). In addition, recent genomic studies revealed an existing mutation in several downstream components of PI3K/AKT pathway[432-434].

1.6.3.5 NF-κB signaling pathway and cancer

The nuclear factor-*kappa*-B signaling pathway regulates many genes involved in cell proliferation, stress response, innate immunity and inflammation [435, 436] and has been found significantly altered in human cancers. NF-κB transcription factor family consists of p50/p105, p52/p100, c-Rel, RelA and RelB controlling the transcriptional expression of hundreds of target genes. c-Rel, RelA, RelB, p50 and p52 form homo- and heterodimers and subsequently shuttle to the nucleus where they bind DNA regulatory κB sites. Inactivated NF-κB dimers are located in the cytoplasm. A variety of extracellular stimuli such as TNF-α, interleukin-1, growth factors, oxidative stress etc. activate NF-κB signaling via phosphorylation and consequent ubiquitination of I-κB by the E3 ubiquitin ligase complex. The liberated NF-κB dimers translocate to the nucleus and activate transcription of target genes [436]. A variety of cancers such as Hodgkin's lymphoma, B-cells and natural killer T-cells lymphomas showed mutations and miss-regulation of NF-κB [437, 438].

1.6.3.6 Wnt signaling pathway

Among the evolutionary most conserved signaling pathways, Wnt pathway is involved in a variety of cellular, embryological and physiological activities from *C. elegans* to humans. From three central branches of this pathway - Ca^{2+}, the planar polarity and the canonical branch, the last canonical branch has been implicated in tumorigenesis. The Wnt pathway consists more than 30 extracellular Wnt ligands [439]. These ligands bind to a receptor and activate a protein called Dishelved (Dsh) which in turn inhibits the Glycogen-Activated Kinase-3 (GSK-3) that targets β-catenin-Adenomatous Polyposis Coli (APC) complex for ubiquitination and proteolytic degradation. Upon Wnt signaling, stabilized β-catenin translocate into the nucleus where it interacts with DNA-binding proteins of the T-cell Factor / Lymphocyte Enhancer binding Factor (Tcf/Lef) family. Tcf/Lef transcriptionally activates proliferation stimulating genes such as *c-myc* and *cyclin D1* [440]. Mutations in APC or β-catenin are frequently found in small intestinal adenocarcinomas and gastric polyps as well as in chronic and acute myeloid leukemia [441].

1.6.3.7 TGF-β signaling pathway

Another evolutionary conserved and most complicated signaling pathway of metazoans, discovered as an antiproliferative signal in tumors is the Transforming Growth Factor-β (TGF-β) pathway. As Wnt, the TGF-β pathway has also three main branches: the SMAD1/5/8, the SMAD2/3 and the TAB/TAK branch. Down regulation or mutation of TGF-β receptors, inactivation of SMAD4 or $p15^{INK4B}$ has been found in pancreatic ductal adenocarcinomas [442] as well as Bone Morphogenic Protein (BMP) is significantly increased in lung carcinomas [443].

1.6.4 Circadian clock, metabolism and Cancer

As mention previously, cancer is a disease in which various genetic alterations result in uncontrolled cell proliferation that is outside the context of normal tissue development. In a majority of tumor types, an accelerated rate of glucose uptake is observed and therefore serves as a reasonable initial point for understanding differential metabolism in tumors. Several studies also revealed a close relationship between circadian rhythms and metabolism. Genes implicated in the metabolic pathways such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase 2 (PCK2, gluconeogenesis), pyruvate kinases (glycolysis), glucokinase (glycogen synthesis), glucose transporter 2 (glucose transport) and HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A, cholesterol metabolism) exhibit a circadian expression pattern. In addition, studies done in animals lacking core clock genes such as *Clock* and *Bmal1* revealed the presence of metabolic defects such as metabolic syndrome or abolished oscillation in plasma glucose and triglycerides [444, 445]. Another example of the circadian regulation of the cellular metabolism by CLOCK was obtained in the case of liver glucokinase whose mRNA levels oscillate in the liver of wild type, but not in *Clock/Clock* mutant liver [446]. Moreover, it is assumed that perturbation in the expression of CLOCK and SIRT1 circadian regulators might be connected with increased cell proliferation in cancers as well as in defects of various metabolic pathways [446, 447]. Circadian clock genes are in general regulated via epigenetic mechanisms of histone phosphorylation, methylation and acetylation. SIRT1, NAD^+- dependent deacetylase sirtuin-1, plays an important role in metabolism and survival by regulating gene expression via histone modification and deacetylation of several proteins. On one hand, SIRT1 has been associated with tumor growth, where deacetylation of p53 leads to its inhibition resulting in reduced apoptosis after genotoxic stress [448]. In addition, SIRT1 can also promote cancer cell

survival by deacetylating the DNA repair protein KU70 that block mitochondrial translocation of BAX and prevent apoptosis [449]. On the other hand, SIRT1 can also act as a tumor suppressor, an effect demonstrated by the impaired DNA damage response in SIRT1 deficient mice [450]. Moreover, ectopic induction of SITR1 suppresses intestinal tumorigenesis and colon cancer *in vivo* [451].

As mentioned above, SIRT1 is a NAD^+- dependent deacetylase. Circadian oscillation of SIRT activity suggested that cellular NAD^+ levels might also oscillate. Indeed, it was shown that NAD^+ levels oscillate and are controlled by the circadian clock. Moreover, beside NAD^+ levels also nicotinamide phosphoribosyltransferase (NAMPT) exhibits circadian oscillations that are regulated by the core clockwork in mice. [266, 267] Given control of SIRT1 deacetylase activity by NAD^+, circadian regulation of NAD+ seems to be crucial mechanism controlling circadian rhythms, metabolism and cell growth. Interestingly, altered levels of NAMPT have been also linked to the metabolic disorders and cancer [452].

Although the direct link of defects in a clock-mediated metabolic pathway and cancer still remains to be disclosed, circadian control of NAD^+ levels suggests an intriguing route. Further studies are needed to uncover the precise function of oscillating SIRT1 and NAD^+ in the regulation of metabolism and tumorigenesis. Determination of this missing part could amplify our knowledge of cancer growth and metabolism as well as it can open the new strategies for chronotherapy.

1.6.5 The nature and extent of inter-individual variation in gene expression

Over the last few decades, exploration of the genetic basis of inter-individual differences in human physiology and behavior has become a 'hotspot' topic, as research teams from different fields have initiated a hunt for genes and signaling pathways linked to the complex traits that relate genome to physiology and behavior. In general, the regulation of gene expression through signal transduction cascades not only plays a prominent role in natural inter-individual variation, but can also affect many aspects of disease susceptibilities and their medical treatment. Fascinatingly, only a handful of major signaling cascades are responsible for this very dynamic regulatory process - the biochemical interactions conducting cellular signaling are universally conserved and very often reused throughout different cell types, so that the same transduction cascade can regulate a variety of biological processes [453, 454].

At a very basic level, the vast array of genetically-determined differences in human behavior and physiology are caused by changes in DNA sequence – so-called "polymorphisms" by which individuals or populations vary. Recent sequencing of multiple human genomes has revealed notably large differences. In fact, it was estimated that about 0.5% variability exists between two haploid genomes [455]. Some of these polymorphisms occur in structural proteins that lead directly to morphological differences in a particular tissue, e.g. in hair color. More globally, however, there is equally great heterogeneity in many signaling pathway components expressed in all tissues. Many such polymorphisms appear in known regulatory and transcribed regions of genes, allowing one to guess at potential functional consequences. Even more occur in genomic domains with yet unknown function. As a whole, these genetic differences not only elicit differences in physiology and behavior, but also inter-individual variation in treatment efficacy and drug toxicity among different patients for many diseases [456, 457].

To identify specific polymorphisms responsible for particular differences, geneticists have used high-density quantitative trait mapping. Starting from a large population of genotyped subjects (typically more than a thousand), the occurrence of a particular trait is quantified in each subject, and then probabilistically related to each polymorphism in the genome. Invention of this technique has revealed hundreds of chromosomal regions possessing genes implicated in several diseases such as Alzheimer's disease [458], diabetes [459-462], obesity [463], asthma [464] or

cancers [465-467]. Thus, genetic mapping offers a powerful approach to discover genes and cellular pathways affecting disease traits and responses to drugs and environmental exposures.

1.6.6 Cell – based approaches to study gene expression variation and human inter-individual differences in a drug response

Unlike the various model organisms that can be repeatedly exposed to drugs or toxins in the laboratory and subsequently phenotyped, the determination of either natural or diseased human inter – individual phenotypes represent substantial limits. Beside ethical and safety issues, there is usually an inability to control different *in vivo* environmental factors as well as there are difficulties to manipulate the *in vivo* system to evaluate biological changes. Due to these restrictions, *in vitro* cell-based approaches became a rational solution to overcome this problem. Various human cell lines have been already used for human disease modeling as well as in preclinical drug development to determine drug-induced cell growth inhibition or cell death or to identify interactions between the target compounds and drug metabolizing signaling transporter proteins or enzymes.

In recent years, there has been a rising utilization of lymphoblastoid cell lines (LCLs) as an accessible and renewable resource for functional genomic studies in humans. Although LCLs were originally established as DNA sources [468], they are now being extensively employed in studies of the genetic and epigenetic determinants of gene regulation [469-473], likewise for the exploration of the host responses to different perturbations or treatments, such as knockdown [474], radiation [475, 476] and drugs [477, 478]. Since the genetic makeup has been shown to play an important role in differential drug response among human individuals, the availability of extensive genotype data for many panels of human LCLs allows performing the study of genetic variants contributing to inter – individual variation in susceptibility to drug [479].

Besides LCLs, for example hepatic cells have been used as a model system to study the pharmacogenetic effects of cytochrome P450 family of enzymes [480]. Additionally, these cells have been used in determining the genetic control of expression and splicing differences in drug –metabolizing genes [481]. Although hepatocytes are very often the most relevant tissue to determine variant gene function in drug metabolizing enzymes for many pharmaceutical agents, this tissue is expensive and moreover difficult to access. Thus, the molecular phenotyping of more accessible cell types such as blood cells, lymphocytes or skin fibroblasts may represent

equally valuable cell – based systems that could be employed in the basic as well as applied research.

Along with the cell lines derived from healthy human subjects, cells obtained from diseased tissues such as NCI – 60 bank of cancer cell lines have shown to be useful models for the investigation of direct effect of a drug on a variety of tumor tissue types. As with LCLs, mRNA expression [482, 483], SNP genotype data [484] and proteomic data [485] are available for these cell lines. Usage of this cell-based disease model has resulted in identification of transcription factors that predict sensitivity to chemotherapeutics as well as proteomic and microRNA profiles predicting drug response [486-489]. Moreover, polymorphisms in candidate genes associated with drug response *in vitro* have been successfully identified as well [490-492]. In addition, a combination of SNP data and gene expression profiles for the panel of tumor cell lines revealed that cytotoxicity to the alkylphospholipid analog *perifosine* is associated with MAPK and apoptotic pathways [493].

The recent revolutionary discovery of induced pluripotent stem cells (iPSCs) [494-497] opened a new avenue of cell – based approaches applicable in drug discovery, *in vitro* disease modeling, as well as in cell replacement therapy. Recent studies demonstrated that the recapitulation of *in vitro* disease phenotype via iPSCs seems to be feasible for numerous monogenic diseases [498-503]. The availability of such a disease relevant pathological cell obtained directly from the patient might definitely benefit the drug discovery. Thus, "a patient in a dish" represented by iPSCs seems to have a promising perspective in the discovery of various drug effects and drug toxicity among the population of patients.

Until now it is clear, that human cell systems could serve as a powerful tool to study human inter-individual differences in a natural as well as disease state. Development of cell – based system that is the most relevant for the phenotype of interest is a challenging task and one of the most significant considerations is whether the chosen cell lines express the relevant pharmacodynamic signaling pathways.

CHAPTER 2

Thesis Aims

Accurately functioning signal transduction pathways, including circadian signaling, are responsible for the human physiology and behavior. Therefore, it's not surprising that mutations in these cascades have been linked to the pathogenesis of different human disorders and cancer. Although the drug targeting of these pathways seems to be useful for a variety of diseases, rising evidence of inter-individual variation in the therapeutic responses becomes a challenge for the development of pharmaceutically effective drugs. Given the role of gene expression in shaping phenotypic variation in health and disease, exploring the nature of causative variation is crucial. In general, a relatively small set of conserved signal transduction pathways is implicated in control mechanisms of the gene expression regulation. The inter-individual differences within these pathways contribute to a variation of gene expression that is in turn responsible for the overall phenotypic makeup of each individual.

The main aim of this thesis was to develop an innovative and possibly high throughput profiling technique that would in future afford the opportunity to perform genetic mapping of the factors responsible for the natural inter – individual variation in circadian (Chapter 3.1) as well as other major non – circadian signaling pathways (Chapter 3.2). This noninvasive cell-based signaling profiling technology showed a potential to give more light into cancer, behavior and pathogenesis (Chapter 3.3). Understanding inter – individual differences in the expression pattern of major signal transduction events will certainly bring more insights into the knowledge of natural human variability regulated by complex operation of these pathways. Especially for the case of disease, coupling gene expression profiling with the results of genome wide association studies could be a promising way forward to unravel the principal mechanisms resulting in disease pathogenesis.

CHAPTER 3

Results

3.1 Inter-individual variation in human circadian rhythmicity

In 2007, *Science* magazine reported it's each year "Breakthrough Of The Year" News – Human Genetic Variation, that was devoted to the latest remarkable advances in understanding the genetic basis of normal human phenotypic diversity and susceptibility to a broad range of diseases. Prior to that in the same year, "the breakthrough paper" published by Levy and his colleagues reported that the human genome, composed of 3 billion nucleotides, varies by 0.5% among individuals [504]. This genetic variation that differs from person to person influences the human phenotypic differences such as eye color, height or disease perceptivity and responses to various drugs. As discussed in Chapter 1 (General Introduction), circadian clocks that are genetically determined, orchestrate many aspects of human physiology and behavior and any genetic or epigenetic alteration of this important timing system leads to a development of various disorders. Given the advances of genomic approaches and conservation of circadian clocks in various peripheral tissues and cells, we decided to develop a technique that would eventually reveal the genetic origins of the human individual differences in circadian chronotype and other behaviors at the cellular level. These studies are described in the following chapter.

Inter-individual variation in human circadian rhythmicity

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BACKGROUND

The circadian molecular pathway is responsible for production and maintenance of daily rhythms in human behavior and physiology and several studies demonstrated that properties of this human circadian signaling pathway differ widely within the population. Moreover, it was also discovered that not only SCN neurons of the central circadian pacemaker but also many other peripheral tissues possess circadian clocks that have similar features as the one found in the SCN. Thus, this duplication opens the possibility to study human differences in peripheral tissues at a cellular level.

METHODS/RESULTS

The initial step of the study was to define the type of human cells (lymphoblastoid cells-LCLs or umbilical cord fibroblast cell line - UCFs) for the measurement of human circadian properties such as circadian period length or phase. Different transfection methods including lentiviral, adenoviral and Herpes Simplex Virus amplicon vector-mediated gene transfer as well as electroporation of an Epstein-Barr Virus-based vector containing the circadian reporter, showed significant circadian gene expression in control cell lines, however, no circadian expression pattern for the LCL and UCFs was obtained. Additionally, the detection and quantification of mRNA for circadian clock genes during a 24 hours cycle revealed only irregular circadian gene expression of low amplitude.

CONCLUSIONS/SIGNIFICANCE

For the measurement of circadian period length, one has to introduce a circadian reporter construct. Human umbilical cord fibroblasts display a lot of advantages such as easy isolation and cultivation, and high amplification potential. On the other hand, lymphoblastoid cell lines (LCLs) with very poor transfectability are commercially available as large human pedigrees, and would therefore be immediately suited to linkage and association studies. However, lack of circadian rhythmicity in both LCLs and UCFs makes these cell lines unsuitable for the mapping of genetic loci responsible for differences in human daily behavior. Despite these results, we still

believe that an adequate library of for example human primary skin fibroblasts, would be a perfect system in which the human phenotype of circadian period length, could be used as a quantitative trait for linkage or association studies, and would certainly provide more insights into the genetic origin of human variation in circadian signaling pathways.

3.1.1 INTRODUCTION

Circadian rhythms represent a biological phenomenon that controls a variety of essential processes in living systems, ranging from bacteria to humans [187, 505]. Beside the body temperature, hormone secretion, cell cycle progression and drug metabolism, the most obvious function regulated by circadian rhythms is the daily sleep/wake cycle in mammals. Many studies showed that human individuals differ in the circadian preferences as well as in their sleep habits. These variations occurring in the daily behavior have been mainly assessed by the questionnaires designed to associate human individuals with the temporal preferences called "morningness – eveningness" [506]. Briefly, a so called morning type person often labeled as "Lark" is someone who gets up easily and exhibits more alertness in the morning than in the evening. On the other hand, "Owl" -- an evening type of person -- is more alert at night and is able to sleep late in the morning. However, most individuals are on the scale position somewhere between the morning and evening-type and are described as neither type [507].

Despite the fact that age, gender or work schedules [508-511] remarkably affect scores/questionnaires, several recent studies revealed an existence of a genetic basis that underlies the trait-like character of the human chronotype. Both twin and family studies suggest that diurnal preference is 50-60% heritable [512-515] and various single nucleotide polymorphisms have been associated with chronotype preference as well as with sleep-wake syndromes. For instance, the first such report described a single nucleotide polymorphism (SNP) in the human 3'flanking region of the CLOCK gene (3111C/T) that showed allele association with eveningness [339, 516]. Moreover, the mutation in *Per2* has been linked to the advanced sleep phase syndrome, potentially by disrupting the target site for *casein kinase 1ε* phosphorylation [517-520]. Two other reports have associated PER3 alleles with delayed sleep phase syndrome, where one rare haplotype represented by a missense mutation was reported by Ebisawa and subsequently associated with diurnal preferences in European population [338, 521]. The other report revealed a variable tandem repeat polymorphism within the *Per3* coding

region in which the shorter allele was linked to delayed sleep phase syndrome. Interestingly, the shorter allele in the normal population was associated with extreme evening preference while the longer allele with extreme morning preference [522]. The three period genes are the main elements of the negative feedback loops. As well as mutations in the *Per2* and *Per3*, the T2434C polymorphism in the *Per1* gene was associated with human extreme diurnal preference. The C allele was more frequent in subjects with morning than in subjects with extreme evening preference [523].

Thus, the importance of circadian core clock genes in the human diurnal behavior is undeniable and their harmonized interplay within the networks of the transcriptional – translational feedback loops became a molecular keystone of circadian biology. Beside the master oscillator, located in the suprachiasmatic nucleus; the expression of core clock genes has also been identified in "the peripheral slave oscillators". Both, master and slave oscillators seem to possess the same circadian molecular makeup what predicts the oscillating peripheral tissues or cells to be a suitable model system to study various aspects of circadian biology.

Until recently, the characterization of human clocks and their genetic diversity was limited by labor – intensive difficulties and expense, since the measurement of human circadian properties under laboratory conditions requires prolonged subject observation. As mentioned previously, a considerable innovation came with the discovery of peripheral oscillators and many mouse studies began to use *in vitro* circadian gene expression to complement behavioral analysis. In these studies, constructs coding the luciferase gene fused to a circadian promoter have been utilized to determine the circadian period length, phase or amplitude in a simple real-time bioluminescent measurement [168, 524]. Moreover, the same technology was applied to measure high-amplitude circadian gene expression in mouse NIH 3T3 fibroblasts and rat fibroblasts [171, 172]. A novel method employing a lentivirally delivered circadian reporter to measure the human circadian rhythms from various cell types was introduced by Brown and his colleagues in 2005 [287]. The developed lentivector construct contains a firefly luciferase gene whose expression is controlled by the promoter and 3'untranslated region of the mouse *Bmal1*circadian gene, flanked by the long terminal repeats. Introducing the so called "enhancer trap decoy" represented by the strong human *Elongation Factor 1α* promoter (*EF1α*) followed by an SV40 transcription terminator resulted in excellent circadian oscillations of the luciferase activity. Besides blood monocytes and hair keratinocytes, easily accessible primary fibroblasts derived from the simple

tissue biopsy showed big potential in the study of human circadian rhythms. Indeed, several recent studies utilized human skin fibroblasts to access the human circadian behavior. For instance, it has been shown that the primary skin fibroblasts derived from healthy individuals could not only predict human diurnal behavior but also the circadian period length measured in these fibroblasts *in vitro* was nicely correlated with the physiological period of tested individuals [288, 289]. Additionally, significant differences in the period length among the human subjects has been reported [287].

Given the genetic origin of circadian variation, conservation of circadian clocks in fibroblasts as well as robustness of the lentiviral reporter technologies suggested exciting routes for my initial study, where for example the period length or phase of daily rhythmicity can be used as quantitative traits in linkage and/or association studies. With advances in genetic mapping strategies, it would be possible to determine genetic modifiers that underlie the human circadian individuality. The success of such linkage or genome-wide association (GWA) studies is strongly dependent on their proper design. The primary concern in this kind of studies is usually focused on the probability of detecting a causal variant, also called the power of the study. The number of individuals used in the study is a crucial determinant of the power. Although the number of subjects used in traditional and expression QTL mapping widely varies from one study to another, it was shown that eQTLs mapping using *in vitro* data need much lower power necessary to carry out GWA studies [525]. For this purposes, various cell banks over the world have been established to meet the requirements of genetic analysis. In this study we used lymphoblastoid cells from the completely genotyped human pedigrees as well as umbilical cord fibroblasts that are conveniently stored as easily accessible cell libraries.

3.1.2 RESULTS

Several transient as well as stable transfection techniques have been developed to introduce foreign DNA molecules into the eukaryotic cells with the aim to study the gene expression. Since lentivirus based vectors, adenovirus vector – mediated reporter system as well as Herpes simplex virus type – 1 amplicon vectors are the most powerful systems able to infect a broad spectrum of dividing and quiescent cells, we employed these technologies to determine the circadian clock gene patterns in Lymphoblastoid (LCLs) and Umbilical Cord Fibroblasts (UCFs) cell lines.

3.1.2.1 Use of lentiviral and adenoviral circadian reporters in LCLs did not show circadian oscillations

Considering the ability to transduce a wide variety of cell types, stable integration and potentially long lasting and heritable gene expression, HIV-1 derived lentiviral vectors offer unique robustness and versatility as natural vehicles for gene delivery. Our laboratory developed a lentiviral reporter construct containing a mouse Bmal1 promoter, the firefly luciferase coding region and the Bmal1 3'UTR, flanked by the long terminal repeats, that allows us to measure human circadian period length directly in the primary skin fibroblasts. Therefore, we employed this reporter system to determine circadian gene expression pattern in a lymphoblastoid cell line. Since lymphoblastoid cell lines show lower transfection levels, simple incubation of the lentiviral supernatant and cells was coupled with a spinoculation method. In this way, lymphoblastoid cells were resuspended in the viral supernatant of two distinct concentrations (10x and 100x) and centrifuged (spin down) in the presence of polybrene - a reagent that was shown to enhance retroviral transfection efficiency. Since only a fraction of cells integrate and express the gene of interest, it is usually necessary to include a selection scheme to isolate positive transformants. Hence, a puromycin mediated selection has been done to obtain the homogenous population of cells carrying the circadian lentiviral construct. Besides LCLs, NIH3T3 mouse fibroblasts have been used as a control cell line. Several days after the lentiviral transduction and antibiotic selection, a real-time bioluminescence measurement revealed no circadian gene expression in LCLs, even when we used different virus concentrations (Figure 1A, B). Although signals were at background levels in LCLs, the antibiotic resistance of the cells proved that infection had indeed occurred.

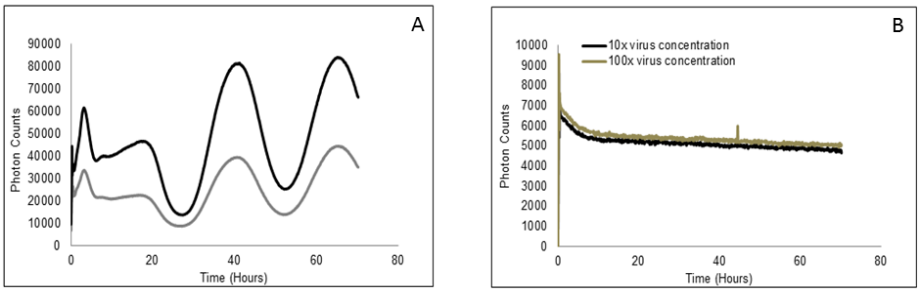


Figure 1 Bioluminescence measurement of the circadian gene expression. A) control NIH 3T3 fibroblasts and B) Lymphoblastoid cell lines after transduction with the lentiviral reporter construct coding the expression of luciferase gene under the control of the circadian Bmal1 promoter. LCLs were transduced with two different virus concentrations: 10x, and 100x. Measurement was done in the presence of luciferin as substrate after 20 min of the synchronization with dexamethasone. Reading time: 1min intervals.

Apart from retroviral reporters, adenoviral vectors also possess several advantages such as large carrying capacity, wide tropism and ability to infect non – dividing cells what predict them to be a reliable system for both *in vitro* and *in vivo* studies. Due to these benefits and the fact that the lentiviral reporter system was insufficient to deliver our reporter construct into LCLs, we employed adenovirus vectors coding the *Bmal1* circadian reporter construct to determine the circadian gene expression in LCL and control NIH3T3 cell lines. Prior to the bioluminescence measurement, both cell lines have been incubated in the presence of adenoviral particles for 24 hours and after transfection, the cells were cultivated for additional 48hours in the normal growth medium. While the control NIH3T3 cell line showed a regular circadian gene expression of expected amplitude, no – circadian pattern of low bioluminescence levels was observed for lymphoblastoids (Figure 2).

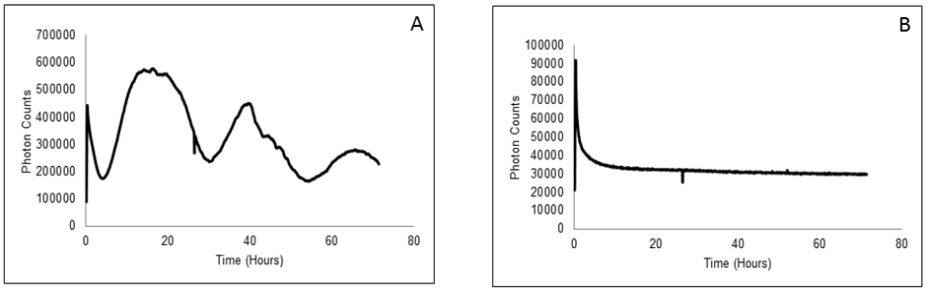


Figure 2 Bioluminescence measurement of circadian gene expression. A) control NIH 3T3 fibroblasts and B) Lymphoblastoid cell lines after infection with the adenovirus circadian reporter construct coding for the expression of luciferase gene under the control of Bmal1 circadian promoter. Measurement was done in the presence of a substrate luciferin after 20 min of synchronization using dexamethasone. Reading time: 1min intervals.

3.1.2.2 Herpes Simplex Virus amplicon reporters showed high infection rates, however no circadian oscillations

Since lentiviral and adenoviral vectors failed to show any circadian gene expression in LCLs, we next tested a DNA virus that belongs to the *Herpesviridae* family - Herpes Simplex Virus amplicon vector (HSV – 1). The usual HSV-1 amplicon consists of a plasmid backbone harboring a bacterial origin of DNA replication plus antibiotic resistance, two non-coding sequences from the wild type HSV-1 genome necessary for replication and packaging of the amplicon into infection particles; and a transgene expression cassette. Due to the absence of viral gene expression, the amplicon is replication defective and its episomal existence results in stable

maintenance in post-mitotic cells. Among many advantages, HSV-1 vectors have an impressive capacity for delivering transgene unit up to 150kb. Moreover, since it does not integrate into the host genome, the conventional amplicon does not lead to insertional mutagenesis.

To determine transfection rates of the gene delivery, LCLs have been initially infected with the HSV – 1 amplicon vector coding green fluorescent protein (GFP) under the control of the cytomegalovirus (CMV) promoter. As previously, cells transfected by Herpes Simplex viral particles were incubated for 24 hours and monitoring of the GFP expression was examined. As shown in Figure 3, the control HEK 293A cell line exhibited 50-80% transfection efficiency and the lymphoblastoid cells showed 20-30% transfection efficiency during 24-48 hours after the transfection.

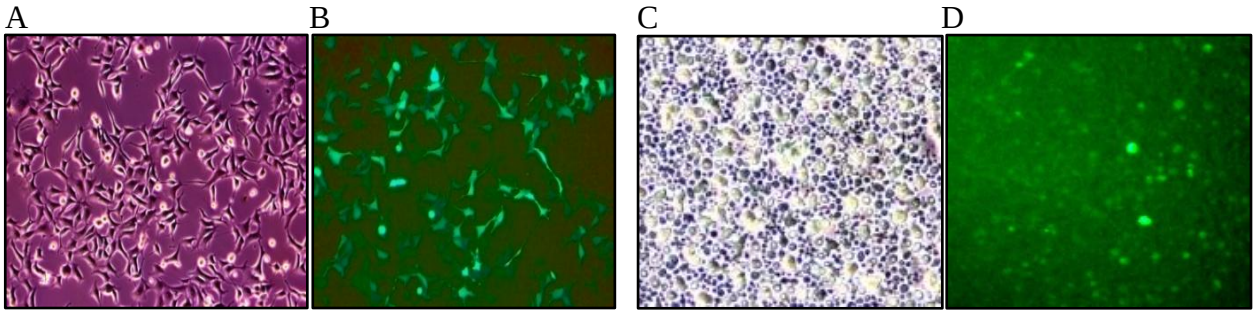


Figure 3 Gene transfer mediated by Herpes Simplex virus (HSV-1) amplicon vectors. A) and B) represent the control HEK293A cell line and C), D) lymphoblastoid cell line 48hours after the transfection with HSV-1 vector coding the expression of green fluorescent protein under the control of CMV promoter.

The promise of this result led us to develop a luciferase version of this vector, where the expression of firefly luciferase gene is driven by the strong CMV promoter. Secondly, the circadian version of HSV-1 vector was prepared by the restriction cloning of the *Bmal1 – luciferase* reporter cassette into the standard amplicon plasmid, pHSVPrPUC. As shown in Figure 4, luciferase expression in HEK293A as well as LCLs reached reasonable bioluminescence levels, although the expression levels have been declining much quicker for LCLs than in the control NIH 3T3 cell line. Consequently, the circadian HSV-1 vector was used to transduce the control U2OS side by side with LCLs. Unfortunately as seen previously, the real – time bioluminescence measurement revealed an expected circadian pattern in the control cell line, however, no circadian gene expression has been observed in LCLs.

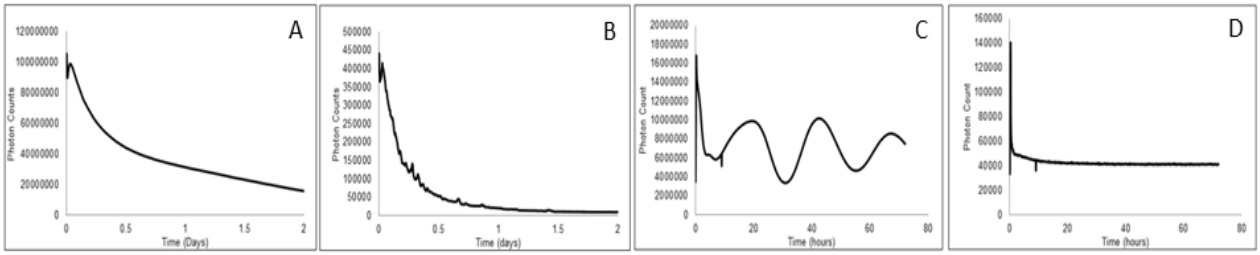


Figure 4 Herpes Simplex Virus - 1 amplicon vector mediated transduction:HSV-1 vector coding luciferase gene under the control of CMV promoter in A) control NIH 3T3 fibroblasts and B) Lymphoblastoid cell lines. Circadian *Bmal1* – luciferase version in C) control U2OS and D) Lymphoblastoid cell line. Dexamethasone synchronization was performed for C) and D) measurement. Real time bioluminescent measurements were done in the presence of a substrate luciferin. Reading time: 1min intervals.

3.1.2.3 Epstein Barr virus – based vectors revealed moderate delivery of a circadian reporter construct but no circadian effect

Altogether, none of the three viral based vectors tested, showed a sufficient transfection efficiency in the lymphoblastoid cell line. Since it is possible that these viruses are becoming rapidly inactivated and consequently the target gene expression is abolished within the LCLs, we tried to overcome this problem by utilization of episomal (extrachromosomal) eukaryotic expression vector that offers an attractive alternative. Such episomal vectors based on Epstein – Barr virus components have found significant and increasing use in molecular biology as well as in biotechnology. In the first step, we cloned the circadian cassette into the pCEP4 episomal mammalian expression vector and as a transcription method the electroporation/nucleofection method has been selected. Cells resuspended in a mixture of the plasmid DNA and buffer solution were exposed an electrical pulse and kept for 24hours in the normal growth medium to recover. After this period, we performed a real-time bioluminescent measurement of the circadian gene expression (Figure 5). The robust *Bmal1* – circadian gene expression was obtained in the control NIH 3T3 fibroblast cell line during seven days of measurement, however, again no circadian gene expression was found for lymphoblastoid cell line.

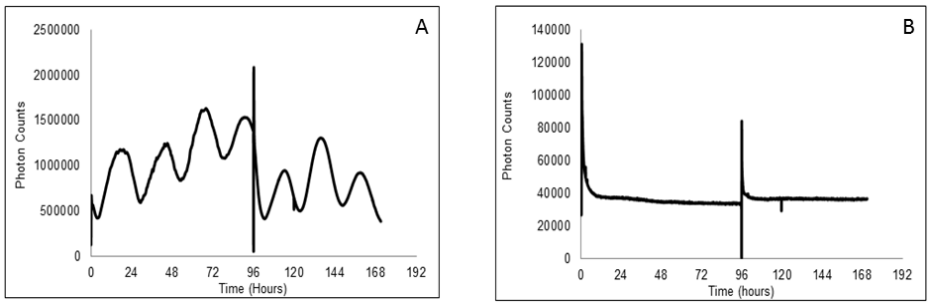


Figure 5 Bioluminescent measurement of nucleofected episomal circadian mammalian vector (pCEP4-Bmal-luc reporter). A) control NIH 3T3 and B) lymphoblastoid cell line during seven days. The synchronization of the circadian phases was performed by dexamethasone treatment over 20 min and luciferin was added as a substrate for the measurement. Reading time: 1 min intervals.

3.1.2.4 Lymphoblastoid cell lines show poor circadian rhythmicity at the level of mRNA

Neither viral integrating nor extrachromosomal eukaryotic reporters showed circadian gene expression in the lymphoblastoid cell line, which suggested an altered or defective circadian clock gene expression within LCLs. To verify this prediction resulting from our datasets, we performed a so called "around-a-clock" experiment, in which the dexamethasone synchronized lymphoblastoid cells have been harvested every four hours during a 24h cycle. Total RNA was isolated and used for quantitative real – time polymerase chain reaction to determine the circadian variation of *Bmal1* and *Rev-Erbα* clock genes within 24 hours. NIH 3T3 fibroblasts that possess strong circadian gene expression were used as a control cell line. Q-PCR results that are shown in the Figure 6, revealed no circadian gene expression in the lymphoblastoid cell line and the expression of *Bmal1* and *Rev-Erbα* circadian clock gene showed rather disturbed and random gene expression patterns with very low amplitude level.

Facing these results, it became clear that lymphoblastoid cell lines seem to have strongly down regulated and altered expression of the circadian core clock genes, which regrettably make them completely unsuitable for our study and the mapping of the genetic modifiers that are responsible for the human circadian variation in the daily behavior.

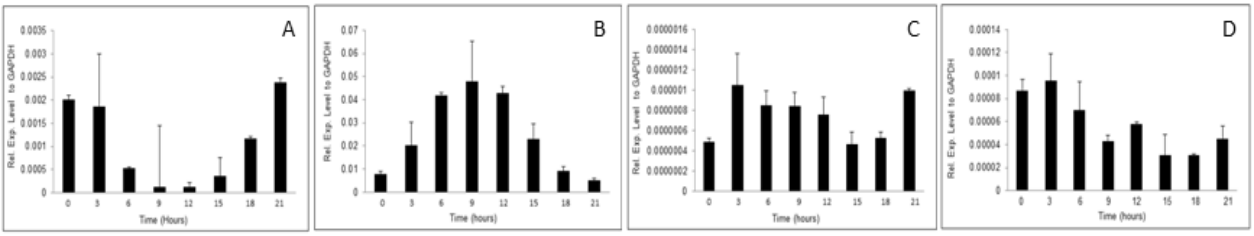


Figure 6 Results of quantitative Q – PCR. A) *Bmal1*, B) *Rev - Erbα* RNA expression from NH 3T3 cell line and C) *Bmal1*, D) *Rev – Erbα* RNA expression from lymphoblastoid cell lines harvested each 3 hours around a clock. Each sample represents the average +/- standard deviation for two replicates for each time point.

3.1.2.5 Umbilical cord fibroblasts show weak circadian clock gene oscillations

For genetic studies of human inter – individual circadian variation, large libraries of the human cell lines are desirable. Although lymphoblastoid cell lines are stored as such a library, we discovered that this cell line is lacking a robust circadian gene expression and is therefore unsuitable to accomplish the aims of our study. Despite this big disappointment, we attempted to utilize the library of umbilical cord fibroblast cell line that has been show to share common characteristics with human skin fibroblasts that are known to possess a strong circadian clock gene expression.

To determine the circadian gene expression in this UCFs cell line, the same strategy as the one used for LCLs was applied. The lentiviral reporter construct coding the circadian expression of firefly luciferase gene was used to transduce this cell line over 24 hours and the real-time photon count measurement was performed after the puromycin selection of the positive transformants (Figure 7). Although umbilical cord fibroblasts showed reasonable levels of bioluminescence expression, circadian expression of *Bmal1*gene was significantly damped in contrary to the robust and high amplitude rhythms obtained in the control U2OS cell line. Moreover, a different synchronization protocol (e.g. high content serum - 50%) didn't show any improvement of the amplitude of these rhythms (data not shown).

To verify this observation, we performed a similar around a clock experiment as in the case of LCLs to determine the levels of mRNA for circadian clock genes in UCFs. Control NIH 3T3 fibroblast cell line side by side with umbilical cord fibroblasts have been synchronized by synthetic glucocorticoid dexamethasone and dishes of cells were harvested each four hours

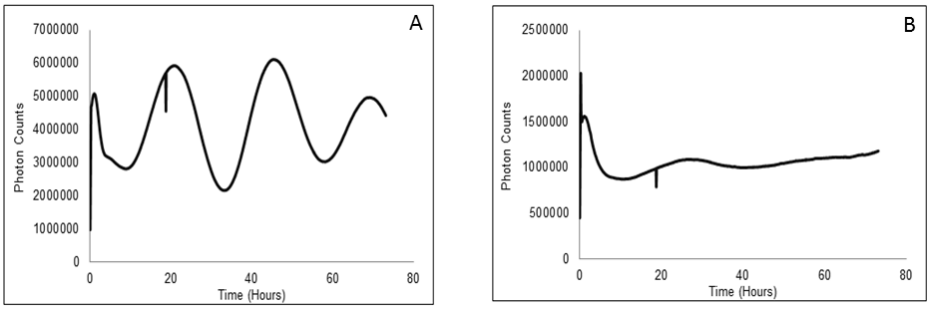


Figure 7 Real-time bioluminescence measurement of the circadian gene expression. A) control NIH 3T3 fibroblasts and B) umbilical cord fibroblasts measured after the transduction with the lentiviral reporter construct coding the expression of luciferase gene under the control of the circadian *Bmal1* promoter. Measurement was performed in the presence of a substrate luciferin after the 20 min of the dexamethasone synchronization. Reading time: 1min intervals.

during 24h hours. Total RNA was then extracted and used for qPCR measurement. As can be seen in Figure 8, umbilical cord fibroblasts showed only random and low level *Bmal1* and *Rev-Erbα* gene expression (Figure 8C and D) when compared to the circadian pattern of the control mouse fibroblasts cell line (Figure 8A and B). As for the lymphoblastoid cell line, the lack of a robust circadian gene expression in UCFs is interfering with our goal to perform the genetic mapping of the loci that underlie the circadian differences within the human population.

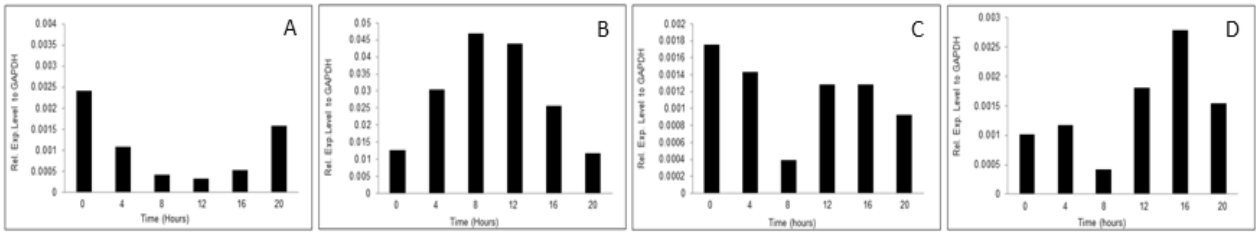


Figure 8 Results of quantitative RT – PCR. A) ***Bmal1***, B) ***Rev - Erbα*** mRNA expression from NH 3T3 cell line and C) ***Bmal1***, D) ***Rev – Erbα*** mRNA expression from umbilical cord fibroblasts harvested each 4 hours around a clock.

3.1.3 DISCUSSION

Human circadian clock, whose activity varies widely among even normal healthy individuals, is responsible for the regulation of human physiology, mood and cognition. Several studies showed that in addition to a "master clock" located in the suprachiasmatic nucleus of the brain, nearly all mammalian peripheral tissues such as heart, liver, lungs, spleen or kidney possess circadian clocks whose molecular set up is in general the same as the one operating in the SCN[168]. Besides these, cell autonomous and self-sustained circadian rhythms have been widely demonstrated in peripheral cells such as human primary skin fibroblasts or keratinocytes [169, 526]. Additionally, various blood cell types possess circadian gene oscillations as well. For instance, human peripheral mononuclear blood cells (e.g. leukocytes, monocytes, macrophages) display circadian properties [527-531] and the functional molecular clock mechanisms has been found in the class of lymphocytes called Natural Killer cells [532, 533] as well as in primary B lymphocytes [534]. Thus, blood beside the skin, is the most accessible and common tissue source at the clinical levels. Therefore circulating leukocytes, lymphocytes or other cycling cells might be an ideal source for the study of the human circadian clock.

The primary goal of this study was to establish a technique and cell lines that would allow us to perform the expression quantitative trait mapping to reveal the genetic modifier that underlie the differences in the circadian signaling pathway among human individuals. For this purposes, suitable number of subjects or better said cell lines derived from the human individuals is necessary. Lymphoblastoid cell lines and umbilical cord fibroblasts are stored as large cell repositories that already contain genome-wide transcriptional information and therefore are perfect for such studies. As mentioned above, several reports showed the existence of circadian rhythms in peripheral as well as human PMBCs including primary B-cells, however, to our knowledge there is no report about the molecular signature of the circadian clock in LCLs or umbilical cord fibroblasts.

Circadian promoter-luciferase reporters have proven to be useful tools for circadian biologists, reporting such basic clock properties as period length, phase, and amplitude via bioluminescent reporting of circadian gene expression [186]. To deliver such reporters into cells for stable sustained gene expression, our laboratory developed a lentivirus-mediated gene transfer that offers a plethora of advantages. Beside the potentially long lasting and heritable gene expression, these vectors have a large packaging capacity and the ability to infect various

dividing and non-dividing cell lines. Since human primary cell cultures as well as LCLs are quite resistant to transient technologies and a large amount of introduced DNA can itself alter gene expression, a lentiviral delivery system seemed a reasonable choice [535]. To our surprise, lentivector mediated gene transfer showed very poor transfectability of lymphoblastoid cells and revealed only slight circadian rhythms with extremely low amplitude in umbilical cord fibroblasts. Experiencing these results, we next tested other known viral vector systems that have been previously shown to be powerful tools for the delivery of reporter constructs into various difficult to transfect cell lines. However, neither adenoviral nor Herpes Simplex Virus amplicon vectors revealed a circadian gene expression pattern of LCLs. At this point, all results suggested that viral vectors tested might be actively repressed within the LCLs and therefore ineffective to show the circadian gene expression or that the circadian gene expression by itself is down-regulated or strongly altered in LCLs. To distinguish between these two possibilities, we employed non integrative Epstein – Barr virus based episomal vectors carrying our circadian reporter cassette. Unfortunately, no circadian gene expression in LCLs besides the normally oscillating control cell line was observed. Since it was unlikely that LCLs are refractory to all transduction techniques, we performed mRNA profiling of the circadian core clock genes. As lymphoblastoid cells also umbilical cord fibroblasts showed only a random, irregular circadian expression of low amplitude when compared to the regularly cycling NIH3T3 control cell line.

Lymphoblasts are immature cells that usually differentiate to form mature lymphocytes. Infection by the Epstein-Barr virus induces transformation of these mature cells into lymphoblastoid cell lines that provide an unlimited source of patient DNA as well as other biomarkers. Although the circadian gene expression of primary B lymphocytes was reported in several studies, our results suggest that this biological phenomenon could have been lost by a simple transformation of B cells. Thought this transformation technique creates easy to maintain highly proliferative lymphoblastoid cell lines, long term cultivated LCLs revealed significant genetic instability with a somatic mutation rate of 0.3% [536] that might for example affect the circadian clock genes function. On the other hand, it was shown that the B lymphoblastoid cell lines have about 2.5-fold enhanced proliferation rates when compared to the T cell lines [537]. Although, in this case we cannot speak about the " cancer cell transformation", higher cellular proliferative rates as well as defects in the cell cycle have been associated with the deregulation of many signal transduction pathways (e.g. MAPKinase signaling, JAK/STAT etc.) likewise

with the alteration of the circadian clocks. One can only speculate that EBV transformation of B lymphocytes might have a more significant effect on the circadian clock gene function as well as the defect in MAPKinase signaling pathway could be reflected into the core clock gene expression. To reveal the basis of altered circadian clock gene function in LCLs would therefore need a separate approach, however, that was not the goal of our study.

Primary cells are a good model to study various aspects of circadian biology. Some features such as circadian period length remain stable even under *in vitro* conditions. However, the measurement of the circadian period length of derived cells is exaggerated when compared for instance to the period length of the SCN neurons. One hypothesis of this phenomenon is the absence of the coupling mechanisms among the peripheral cell's oscillators [287, 538]. The discovery that the human umbilical cord fibroblasts lack the circadian gene expression still remains unclear to us. These cell lines do not undergo any transformation procedure and their isolation from the umbilical cord is quite straightforward. Both, morphologically and based upon the expression of the cell surface markers or collagens, UCFs share common similarities with the human primary skin fibroblasts. Since our results showed that these cell lines are able to be transduced by the lentiviral reporters (according to the high levels of photon counts during the measurement), the dampening or very low circadian amplitude rhythms could be due to loss of synchrony among these cells. Various synchronization options (besides those tested) as well as single-cell analysis of bioluminescence would help us to distinguish if the UCFs circadian rhythms are due to a gradual loss of synchrony among independent oscillators or dampening of individual cellular rhythm, if any circadian rhythms exist within this cell lines.

To conclude, our laboratory possesses a powerful lentiviral reporter system that allows us to study human inter-individual circadian variation among the human population. However, the libraries of LCLs or umbilical cord fibroblasts are unsuitable to study these differences between human individuals. A collection of for example human skin primary fibroblasts, which have been already used for the study of human circadian rhythms, would meet the requirements for the expression quantitative trait studies of the human daily behavior.

3.1.4 MATERIALS AND METHODS

Cell culture

Lymphoblastoid cell lines as well as human primary fibroblasts have been purchased from the Coriel Institute for Medical Research, Camden, New Jersey. Such LCLs were established by Epstein – Barr Virus transformation of peripheral blood mononuclear cells using phytohemaglutinin as a mitogen. RPMI 1640 growth medium containing 2mM L-glutamine and 15% fetal bovine serum was used to cultivate this cell line in T25 tissue culture flasks with 10-20ml medium upright position at 37ºC under 5% carbon dioxide. Human primary fibroblasts have been established by outgrowth of undifferentiated mesodermal cells from a biopsy. The medium to culture these fibroblasts was DMEM containing 20% fetal bovine serum, 1% Penicillin/Streptomycin, 1% gentamycin.

Vector production and virus preparation

Lentiviral circadian reporter construct carries the *Bmal1 – luciferase* reporter cassette as previously described in [287]. This cassette has been initially inserted via restriction cloning into the pENTR4 gateway vector replacing ccdB gene located between two *attL1* and *attL2* sites for site – specific recombination of the entry clone with Gateway destination vector. Subsequent recombination reaction between the pENTR4 – *Bmal1 – luciferase* clone and a gateway destination vector pLDEST-Puro was performed by using Gateway® LR Clonase® II reaction. Adenoviral circadian reporter carries an identical *Bmal1 – luciferase* reporter cassette that has been described previously for the lentivirus based vector [287, 288]. Viruses were prepared as described previously [539].

Herpes Simplex Virus type 1 amplicon vector for our experiments was prepared by the introduction of *CMV-GFP*, *CMV-Luciferase* and *Bmal1 – luciferase* reporter cassette into the pHSVPrPUC vector via restriction cloning. The virus production was performed as described in Fraefel et al., 1996 [540].

Preparation of the circadian variation of the episomal expression vector was done by restriction cloning. pCEP4 episomal vector purchased from Invitrogen (Cat.no V044-50) was digested by SalI restriction endonuclease and via blunt end cloning the *Bmal1 – luciferase*

reporter cassette was inserted into the vector. The verification of the correct orientation of the insert was performed and the plasmid was amplified for the nucleofection procedure.

Transfection of the LCLs and UCFs

Lentiviral transfection of LCLs: $1x10^6$ cells resuspended in 1ml of warmed lentiviral supernatant in the presence of 200mM HEPES and 4µg/ml polybrene. Cells were spin down for 90 minutes at 2500 RPM at RT. After the spinoculation, virus was removed and the cells were resuspended in the fresh warm RPMI 1640 medium containing 10% FBS and 2mM L-glutamine and incubated 48 hours at 37°C, 5% CO_2. Lentiviral transduction of UCFs was performed on the semi confluent 35mm Petri dishes containing $2.5\text{-}5x10^5$ cells. Cells were incubated for 6-25hours and the viral supernatant was aspirated. Cells were washed twice with warmed 1x PBS and the fresh growth DMEM medium containing high glucose, 20% FBS, 1% Penicillin/Streptomycin, 1% gentamycin was added. Four day after the transduction, antibiotic selection with the presence of 2ug/ml Puromycin was initiated. Another three day after the selection, cells were ready for the measurement.

Adenoviral transfection was performed similarly to lentiviral transduction, without the spinoculation. Briefly, cultures of LCLs and UCFs were incubated for 6-24 hours with adenoviral supernatant. After this incubation, cells were washed twice with 1xPBS and cells were cultivated next 48-72 hours within the normal growth medium and the bioluminescence measurement was performed.

Nucleofection of pCEP4 - *Bmal1 – luciferase* plasmid was performed by using and optimized T16 nucleofector program. Shortly, 1x107 of lymphoblastoid cells were mixed with 5µg of plasmid in 100µl of the nucleofector buffer V and placed into the electroporation cuvette. After the application of the optimized strength of the electrical pulse (T16 nucleofector program), cell were immediately transferred into the 2ml of prewarmed RPMI 1640 growth medium containing 15% FBS, 1%, 1% Penicillin/Streptomycin, 1mM Sodium Pyruvate. Cells were cultivated during the next 48hours at 37°C, 5% CO_2.

Real-time bioluminescence measurement of the circadian gene expression

After the transduction or nucleofection as described above, circadian rhythms in cell population were synchronized with a synthetic glucocorticoid dexamethasone for 20 min or with the high content of 50% FBS for 30 min or one hour. Medium containing dexamethasone was

replaced with 1x PBS and cells were washed twice. 3-5 days of real time bioluminescence measurement was done in the normal culture medium lacking phenol red but supplemented with the 0,2mM substrate luciferin and 25mM HEPES as described previously [172]. Data were analyzed using Microsoft excel and/or the Lumicycler Analysis program (Actimetrics).

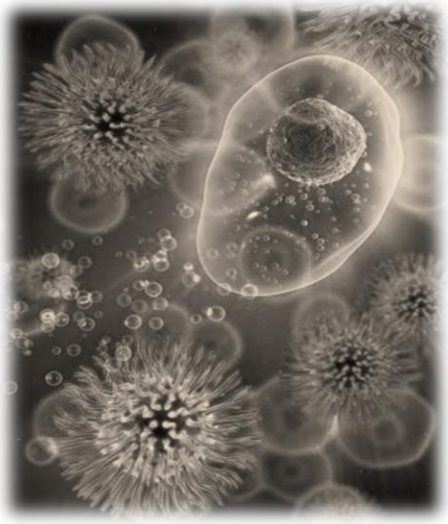
RNA isolation, cDNA production and Quantitative RT-PCR

Total RNA was extracted as described previously in [541]. 0.5µg of RNA was transcribed to cDNA with SuperScript II (Invitrogen) using random hexamer primers according to the manufacturer's instruction. For quantitative real-time PCR 20ng of cDNA was used with the Taqman PCR mix protocol (Roche) using AB7900 thermocycler. Primers used for the detection ale listed in the table below.

Gene	***Orientation***	***Sequence***
Bmal1	*Sence*	GAAGACAACGAACCAGACAATGAG
	Antisence	ACATGAGAATGCAGTCGTCCAA
	Probe	TGTAACCTCAGCTGCCTCGTCGCA
Rev-Erbα	*Sence*	TCACCTGGCAACTTCAATGC
	Antisence	CCTGATTTTCCCAGCGATGT
	Probe	AACCATGCATCAGGTAGCCCTCCAGC
Per1	*Sence*	CGCCTAACCCCGTATGTGA
	Antisence	CGCGTAGTGAAAATCCTCTTGTC
	Probe	CGCATCCATTCGGGTTACGAAGCTC
Per2	*Sence*	GGGCAGCCTTTCGACTATTCT
	Antisence	GCTGGTGTCCAACGTGATGTACT
	Probe	CATTCGGTTTCGCGCCCGGG
Cry1	*Sence*	CACCATCCGCTGCGTCTAC
	Antisence	AGCAAAAATCGCCACCTGTT
	Probe	CGCCGGCTCCTCCAATGTGG
Cry2	*Sence*	CCCGCCTGGATAAGCACTT
	Antisence	AGAGACAACCAAAGCGCAGGTA
	Probe	ATGAGAGACCCCGAATGAACGCCAAC
GAPDH	*Sence*	CACATGGCCTCCAAGGAGTAA
	Antisence	GTGAGGGTCTCTCTCTTCCTCTTGT
	Probe	TGGACCACCAGCCCCAGCAAGA

3.2 Profiling inter-individual differences in human cellular signaling

PREFACE: The circadian molecular pathway is only one of many signaling cascades responsible for the regulation of human physiology and behavior. The same technologies developed for the circadian clock could equally be applied to these other cascades. Therefore, during the last few years, we have extended the focus to other major signal transduction pathways, including MAP-Kinase pathways, the inflammatory responses (IFN-γ), immune system responses (NFAT and NFκB) as well as apoptosis (p53). At a cellular level, nearly all drugs act upon so-called "signaling cascades" of regulatory proteins that transmit information from the cell surface in order to elicit cytoplasmic or nuclear changes. Therefore, the widespread problem of inter-individual variations in responses to many classes of drugs actually begins here: due to genetic variation, the expression or structure of signaling cascade proteins can differ from one person to another. Understanding the cellular determinants of these differences is an important question for pharmacology, and is the basis of "personalized medicine." The following chapter of this thesis called "Profiling inter-individual differences in human cellular signaling" mainly focuses on the development and the utilization of a novel high-throughput viral system that is capable of measuring differences in the output of several signaling cascades among primary fibroblasts derived from different individuals.



Adapted from http://esciencecommons.blogspot.com/2011/09/biochemical-cell-signals-quantified-for.html

Profiling inter-individual differences in human cellular signaling

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BACKGROUND

Basic intracellular signaling pathways are highly conserved among different cell types. Signaling defects have been implicated in many types of human disease, and genetic differences in signaling pathways are presumed to be responsible for inter-individual differences in patient response to many classes of drugs. Nevertheless, elucidating the molecular basis for these differences at the level of individual patients remains a major challenge for medicine.

METHODS/RESULTS

We have developed a novel lentivector-based system to profile drug-induced activation of major signaling pathways in human cells. Using this system, we examined the activation profiles of three principal signaling pathways in fibroblasts from ten healthy human subjects. Surprisingly wide inter-individual differences were observed in all pathways, ranging from fivefold to twentyfold in outliers. These differences were matched by genome-wide differences in transcription from promoters known to be activated by these pathways, as well as by differences in cellular toxicity and efficacy of pathway-specific drugs. Fibroblast profiling of CREB signaling was used to predict the degree of suppression of the hormone melatonin by light in human volunteers, demonstrating the applicability of cellular signal profiling to more complex responses in non-accessible tissues.

CONCLUSIONS/SIGNIFICANCE

We show here that the amplitude of drug-induced signaling varies widely in human fibroblasts, and can be easily profiled using lentiviral reporters. The resulting information can be a potent predictor of both simple drug efficacy and of more complex responses. We suggest that individual fibroblast profiling could be useful both clinically to determine optimal treatments for different individuals, and genetically to map human modifier loci for drug response.

3.2.1 INTRODUCTION

Within individual cells, complex information flow is operated by highly organized signal transduction pathways. Such a pathway typically begins by the binding of a large repertoire of extracellular signaling stimuli to the cell surface receptors. The main function of these mostly membrane-integral receptor proteins is to conduct and integrate incoming signals to a variety of cytoplasmic transducers and amplifiers. These in turn stimulate the activity of various effector proteins, and ultimately evoke essential cellular responses ranging from cytoskeletal changes to nuclear transcription.

Accurate and rapid translation of information from the cell surface to the nucleus plays an important role in fundamental cellular homeostasis. Thus, not surprisingly, mutations in these cascades have been associated with the pathogenesis of various human disorders and the development of different types of tumors. Drugs targeting these pathways have proven therapeutically useful for a wide variety of diseases.

One of the most interesting findings in the last decade was that only a few major signal transduction cascades, each conserved among many different cell types, are responsible for this sophisticated intracellular communication. Although the initial sensor or receptor can vary among cell types, and the activated genes also differ according to local epigenetic modifications and the availability of co regulatory factors, the "cytoplasmic part in the middle" is the same. For instance, one can express a photoreceptor and an ion channel in a kidney cell, and the result is that the kidney cell depolarizes like a retinal ganglion cell in response to light [542]. Another example is the circadian molecular pathway, responsible for nearly all aspects of behavior and physiology [543-545]. Although a core clock oscillator located in the suprachiasmatic nucleus (SCN) of the brain hypothalamus orchestrates the generation and maintenance of circadian behavior ([546, 547], the cellular mechanism of this clock is in fact conserved in most other cells of the body: many studies have revealed that not only the SCN but also other peripheral tissues such as kidney, liver or even skin cells exhibit circadian functions [167, 548, 549], and the operating molecular clock mechanism seems to be the same as the one in SCN neurons [550].

Even if signaling cascades themselves are highly conserved among different cell types, an increasing number of studies show that inter-individual genetic variations can alter these pathways considerably from one person to another. Recent sequencing of multiple genomes have revealed variation of about 0.5% between two genomes [455]. Therefore, understanding these

alterations probably holds the key not only to understanding differences in human behavior and physiology, but also to deciphering the reasoning behind variations in patient responses to many classes of drugs.

Unfortunately, because the functional consequences of most genomic variations remain unknown, the tools to analyze these differences remain relatively crude, especially in human beings. Very few tissues are readily available. Nevertheless, the study of signaling pathways and genetic differences within available tissues has repeatedly proven diagnostic of wider physiological differences. For example, study of the circadian clock properties in human skin fibroblasts has proven diagnostic of diurnal behavior (chronotype) [551]. More broadly, most genetic alterations in cellular processes are generally reflected in the expression signatures of many genes, and therefore can be characterized by gene expression profiling. By this technique, genetic contribution to inter-individual variation in gene expression has been associated with diseases such as breast cancer or diabetes [552, 553].

Nevertheless, the complexities of intracellular signaling – including crosstalk among many different pathways – has rendered the task of quantifying such differences difficult. For this reason, various *in vitro* cell-based reporter assays have been used in attempts to reveal the fundamental role of signaling pathways in health and disease [554-556]. In contrast to a traditional ‘’end-point’’ DNA microarray approach, they provide an accurate functional activity of targeted genes in a cellular and physiological context. However, introducing these reporters into cells in a high-throughput nondisruptive fashion has proven a barrier to widespread application of such technology.

For this purpose, the use of viral systems can provide an ideal solution. Because of their ability to transduce a wide variety of dividing or quiescent cells, and their stable integration and potentially long lasting and heritable gene expression, HIV-1 derived lentiviral vectors offer unique robustness and versatility as natural vehicles for gene delivery [557-562]. For example, the use of lentivirally delivered reporters to study inter-individual differences in circadian clock function in human fibroblasts has revealed that circadian period length in these cells extensively varies among human individuals and furthermore, that individuals classified as early chronotypes have in general shorter circadian period lengths than individuals of late chronotype [563], as has been established in more laborious behavioral studies. In fact, there is a direct correlation

between the physiological period length measured *in vivo* and the circadian period length obtained from human skin fibroblasts [564].

Similar technologies could easily be used to study inter-individual genetic differences in other conserved signal transduction pathways, and could provide a more reliable readout than conventional expression profiling. In this paper, we describe the development of a set of lentivirus-based reporters which allows one to monitor the transcriptional endpoints of major signaling pathways directly in living human primary fibroblasts in real time over multiple days. Besides interesting kinetic patterns, we demonstrate that the peak activation of CREB, ELK1 and CHOP in response to a specific drug stimulus significantly varies among fibroblasts from healthy human individuals. Moreover, we show that these variations in signaling expression pattern are correlated with the transcriptional changes genome-wide, as well as with cellular responses to several pertinent drugs. Finally, we show that signaling responses in one human pathway measured in fibroblasts, CREB, correlate with the more complex physiology of melatonin signaling – which is known to be CREB-dependent from rodent studies. Therefore, our observations suggest that the signals obtained from our potentially high-throughput cell based reporter system could serve as valuable biomarkers for individual therapeutic response and physiological function.

3.2.2 RESULTS

3.2.2.1 Lentiviral reporters respond dose-dependently to pharmacological activators in primary cells.

We have developed a lentiviral cell-based reporter system which allows us to profile drug-induced transcriptional activation of major signal transduction pathways directly in living human primary fibroblasts. This system consists of a set of VSV-G pseudotyped lentiviral vectors (Figure 1A). The first virus is a *Reporter Virus* carries a synthetic minimal promoter with five tandem repeats of the yeast GAL4 binding site that controls the expression of the firefly luciferase gene. This virus also contains an antibiotic resistance marker so that homogenously infected populations of cells can be obtained. The second virus is a *Specific Activator Virus* containing the human cytomegalovirus (CMV) immediate early promoter driving expression of a GAL4 DNA binding domain fused with a pathway-specific transcriptional activator (CREB,

Elk1, CHOP, c-jun, NFAT etc.). The resulting fusion protein is universally expressed in transfected cells, but only transcriptionally active when phosphorylated by the pertinent cellular pathway. The third virus is a *Normalizing Control Virus* encoding the secreted alkaline phosphatase (AP) gene under the control of the CMV promoter. Co-transduction of mammalian cells with these lentiviruses allows specific activator-dependent luciferase signal to be expressed as a ratio relative to AP, thereby controlling for pathway-independent effects such as infection efficiency, toxicity, etc.

In total, reporters were designed for eight major signal transduction pathways: CREB, ELK1, CHOP, NF-KB, p53, c-JUN, NFAT, and SRE. In a first test, each of these was transduced into primary human skin fibroblasts, which were then treated with a pharmacological agent known to activate the pertinent pathway, and bioluminescence was then measured over several days via real-time bioluminometry in the presence of luciferin. Our initial test for these chosen signal transduction pathways showed interesting individual kinetic patterns of pathway-specific transcriptional activation in response to specific drug stimuli (Figure 1B). For our further analyses, we decided to concentrate upon three important signal transduction pathways: CREB, essential for cAMP-dependent G-protein-coupled signaling from a wide variety of receptors; ELK1, a mitogen-activated protein kinase (MAP kinase) important in cell growth signaling; and CHOP, known to be responsive to endoplasmic reticulum stress and involved in DNA damage, growth arrest, and apoptosis. For each pathway, we next determined non-toxic, sub-maximal concentrations of a drug stimulus which would be used in subsequent experiments (Figure 1C). Each pathway showed dose-dependent responses to its pertinent drug. For example, a 5µM concentration of the diterpene forskolin, commonly used to raise levels of cyclic AMP, was determined as sufficient to induce CREB phosphorylation in our assay. 100ng/ml concentration of Phorbol 12-myristate 13 – acetate (PMA), a structural analog of diacylglycerol able to activate PKC, was chosen to induce ELK1 signaling pathway. Finally, 10ng/ml of methyl methanesulfonate, an alkylating agent and carcinogen, was used to activate the transcription factor CHOP.

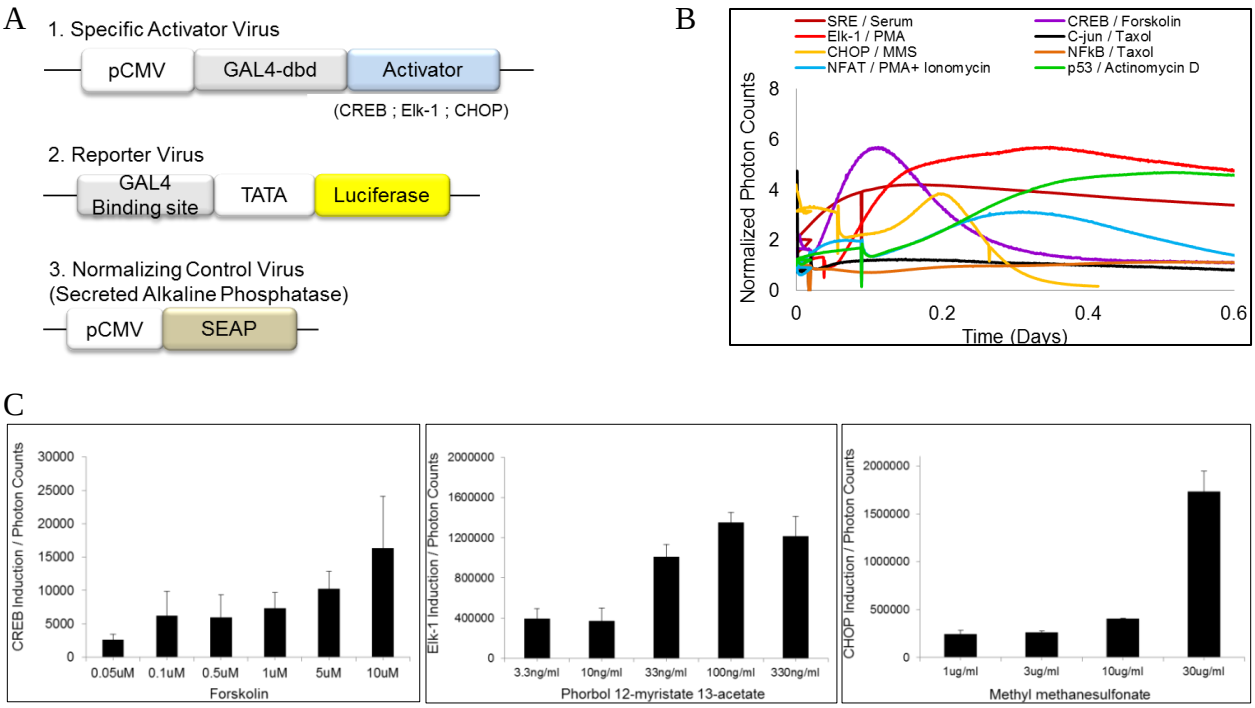


Figure 1 A. Lentiviral cell based reporter system. Specific Activator Virus (1) expresses a chimeric pathway-specific transcriptional activation domain fused to the GAL4 DNA binding. Reporter virus (2) contains the firefly luciferase gene under the control of a minimal synthetic promoter with GAL4 DNA-bdining sites. Normalizing Virus (3) expresses secreted Alkaline Phosphatase. **B.** Pathway-specific transcriptional activation in human primary fibroblasts responds to specific drug stimuli. Raw bioluminescence profiles of human fibroblasts infected with pathway specific reporters, and then treated at time 0 with pathway-specific activating compounds. Drugs used for activation: CREB- Forskolin(5μM); Elk1- phorbol 12-myristate 13-acetate (PMA, 100ng/ml)); c-JUN- Dexamethasone(5μM); CHOP- methyl methanesulfonate (MMS, 10ng/ml); NFAT- phorbol 12-myristate 13-acetate (PMA)/ calcimycin (100ng/ml); NFkB-human tumor necrosis factor α (hTNFα, 50ng/ml), GAS- human tumor necrosis factor α (hTNFα, 50ng/ml); p53- Actinomycin D(5nM). **C**. Dose response curves for the specific drug stimuli used to activate CREB, ELk1 and CHOP signaling pathways.

In order to be useful, a viral reporter system should be robust to most changes in infection efficiency, viral titer, or cell confluence, passage, and health, and should be uniform across different biopsies from the same individual. (Of course, since some of these factors themselves influence intracellular signaling, not every pathway should be robust to all of them!) We systematically tested each of these factors for each pathway, and showed that measured pathway induction – expressed as a ratio of luciferase to AP – was uniform under these conditions. Although factors such as different biopsies, cell confluence at the time of the transduction, or

passage number dramatically changed raw luciferase signals, they had no significant effect when normalized to signal from the AP vector (Supp. Figure 1).

3.2.2.2 Signaling pathway amplitude varies widely in fibroblasts from different individuals

Having our lentiviral cell based reporter system, we were interested if the activation profiles of these three basic signaling pathways measured in skin fibroblasts differ among ten healthy young subjects. To measure inter-individual differences in the expression levels of CREB, ELK1 and CHOP in primary human fibroblasts, semi-confluent dishes of primary skin fibroblasts, each corresponding to an individual subject, were transduced with lentiviral reporters and signals were measured and normalized as described in Materials and Methods. In addition to pathway-specific kinetic patterns, we found surprisingly large inter-individual differences in the peak expression of these tested pathways. Nearly 10-fold differences were obtained in CREB induction between the most extreme subjects (in response to identical 5uM concentrations of forskolin), 5 - fold differences for Elk1, and 20-fold differences for CHOP induction among the tested subjects (Figure 2). Importantly, subjects showing differences in pathway expression varied from pathway to pathway, thereby arguing against systematic bias. To assure that obtained differences in expression patterns were not due to a differential degree of transduction or a possible variability in virus titer, we performed further measurements of "outlying" subjects (showing maximal and minimal induction) for each pathway, and showed that differences were well-preserved across four different concentrations (1x, 5x, 10x and 25x) of the lentivirus used for the experiment (Supp. Figure 2).

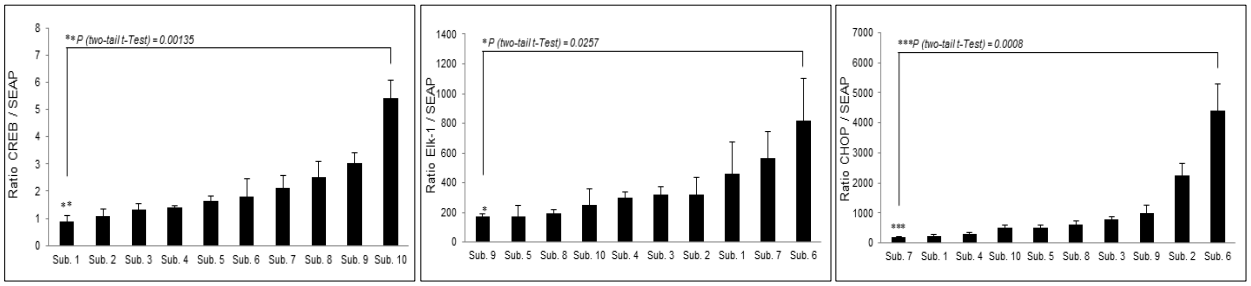


Figure 2 Fibroblasts from different individuals show widely varying induction of different pathways. Fibroblasts from ten subjects were infected with pathway-specific reporters for CREB (**A**), Elk1 (**B**), or CHOP (**C**), and then identically stimulated with 5μM Forskolin (**A**), 100ng/ml PMA (**B**), or 10ng/ml MMS(**C**). Peak activation for each subject is plotted as a ratio to constantly-expressed alkaline phosphatase in the same cells, with the average /- standard error from four replicates in two separate measurements. Approximately 10-fold differences for CREB, 5-

fold differences for Elk1 and 20-fold differences for CHOP were obtained among the most extreme subjects, who were different for each pathway. The probability by Student's t-Test that the most different individuals (CREB – Subject 1 vs Subject10, Elk1 – Subject 8 vs Subject 6, CHOP – Subject 7 vs Subject 6) have the same expression is : CREB/ P=0.0013 , Elk1/ P= 0.025 and CHOP/ P= 0.0008. (*P<0.05, **P<0.01, ***P<0.001)

3.2.2.3 Reporter-based variations in pathway signaling are mirrored by genome-wide transcriptional variation

The pathway-specific inter-individual differences that we observe were significantly larger than what other studies have shown for microarray-based gene expression. Therefore, we next compared the results from our pathway profiling with corresponding experiments performed via microarray-based transcriptome analysis of the same cells harvested at the time of maximal observed induction for each pathway. Using published lists of human genes activated by each pathway (as identified by chromatin expression and microarray analyses by others) [565, 566], we found notable differences in the gene expression profiles of CREB, Elk1, and CHOP target genes between subjects identified as "high inducers" and "low inducers" for each pathway. For example, looking at the expression profile of CREB target genes, we found that forskolin evoked an average 4-fold difference in activation of the fifty most-induced CREB target genes among the "high" and "low" subjects (red vs. black bars, Figure 3A). The same strategy to analyze the data was adapted for differential expression of Elk1 and CHOP target genes [565, 566]. This approach revealed the 3 – fold difference and 2.5 – fold difference in transcription from promoters known to be activated by Elk1 and CHOP pathway, respectively (Figure 3B, C.) Where suitable antibodies could be purchased, these differences also corresponded with inter-individual variations in the levels of the endogenous transcription factors: not surprisingly given the transcriptomic data, we found that that the levels of phosphorylated CREB as well as phosphorylated Elk1 differ among these individuals (Supp. Figure 3).

Importantly, no difference was seen in the average expression of the full chip gene set (Figure 3D). Surprisingly, when standard differential expression analyses (GeneGO, KEGG) were conducted upon these gene sets, in each case the activated pathway was not identified as significantly different among subjects, and in fact was not even identified as one of the principal induced pathways when compared to microarray datasets from uninduced cells (Supp. Datasets

1-3). Stated another way, crosstalk between signaling pathways introduced a level of noise in the data that made direct conclusions from raw microarray data difficult.

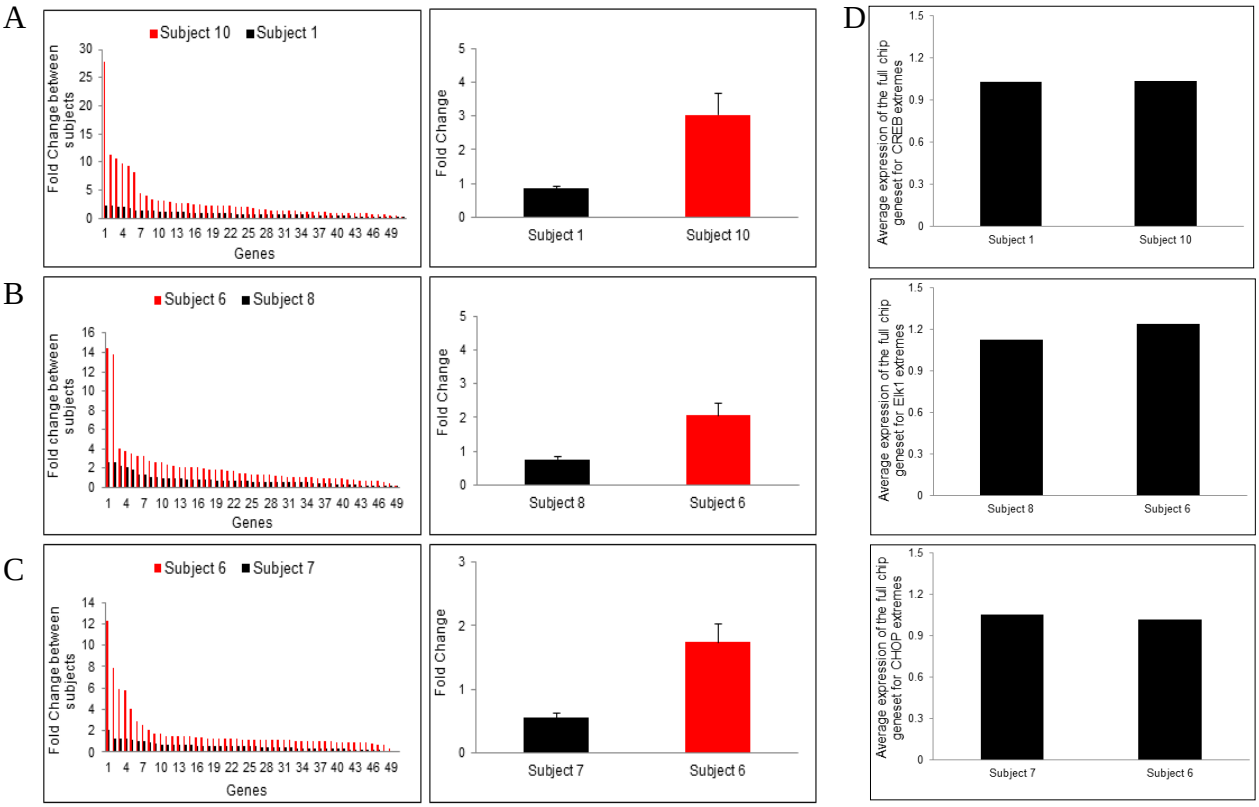


Figure 3 Microarray analysis shows fibroblast transcriptome-wide differences in pathway-specific gene targets among extreme subjects. Whole-transcriptome analysis was performed on cells from the indicated subjects, treated as in Figure 2. For **A)** CREB, **B)** Elk1 and **C)** CHOP, fold-activation of the 50 most activated genes for each pathway are plotted for the subject identified as maximally inducing (red bars, left panel) and minimally inducing (black bars, left panel). Average differences in induction of all pathway-specific genes induced more than 2x is shown in the right panel. For each case, single-dye gene expression microarray analysis was performed in three technical replicates. **D**) No differences were seen among subjects in whole-transcriptome-wide expression from the same microarray datasets.

3.2.2.4 Inter-individual differences in pathway signaling correspond to cellular differences in drug toxicity or efficacy.

Alterations in the extracellular environment cause changes in gene expression levels which in turn lead to physiological responses. Understanding the variation in the gene expression patterns within human populations could provide important insights into the basic phenotypic diversity;

likewise, it could be useful to better interpret the pattern of gene expression variation in disease. To demonstrate the relevance of our findings for drug toxicity and efficacy, we looked at cytotoxicity mediated via CHOP phosphorylation. CHOP, a small nuclear protein also known as DNA damage gene 153 (GADD153), induces growth arrest in response to various extracellular stresses such as UV radiation, endoplasmic reticulum stress or alkylating reagents [567]. We tested methyl methanesulfonate (MMS), an alkylating agent inducing phosphorylation of CHOP and DNA damage [568]. As predicted, the cells corresponding to the subject with the higher CHOP expression level showed decreased cellular viability after MMS treatment (Figure 4B) compared to untreated controls (Figure 4A).

A B

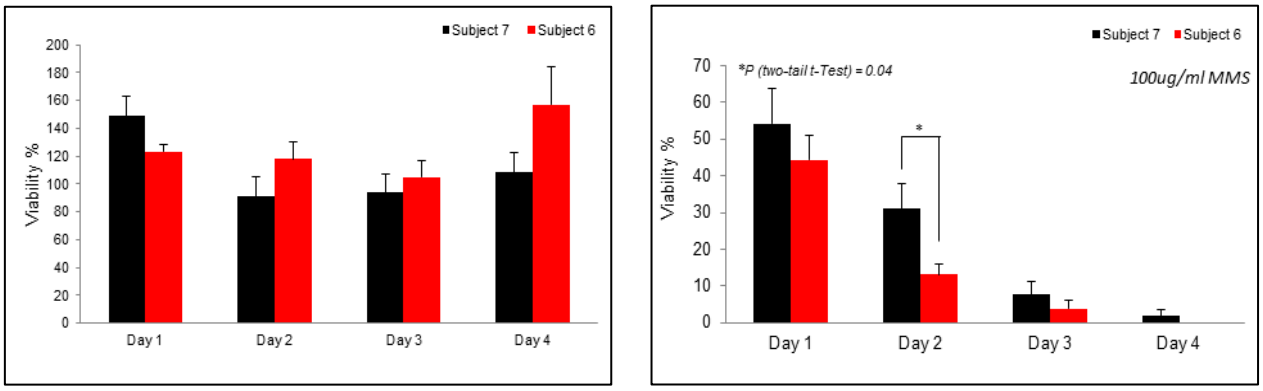


Figure 4 Cytotoxicity mediated via CHOP phosphorylation differs among extreme subjects. Human primary fibroblasts from the subjects exhibiting the lowest and the highest expression level of CHOP were treated with the toxic concentration of methyl methanesulphonate (100ng/ml) during several days and viability was measured via Trypan blue staining each day. The cells corresponding to the subject with the higher CHOP expression level showed decreased cellular viability after MMS treatment (**B**) compared to untreated controls (**A**). (Student's t-test, P= 0.04.) (*P<0.05, **P<0.01, ***P<0.001)

3.2.2.5 Inter-individual differences in cellular pathway signaling correspond to physiological differences in human neuroendocrine response

Our results also point to unsuspected variability in the amplitude of CREB-dependent signaling in primary fibroblasts from different human subjects. It is well-known that this pathway is conserved in many different tissues, where it regulates a variety of processes. For example, in the brain CREB signaling has been found to be involved in the control of melatonin. Melatonin is a hormone of the pineal gland whose circadian secretion is regulated via

multisynaptic neuronal pathway coming from the SCN. At the molecular level, the circadian secretion of mammalian melatonin begins by the adrenergic innervation of the pineal gland, followed by the binding of the norepinephrine to a β_1 – adrenergic receptor and activation cyclic AMP (cAMP) - signaling pathway[569] which through CREB modulates the level of the rate-limiting AA-NAT enzyme [197, 570, 571]. Nocturnal light results in inhibition of this cascade, giving rise to the well-studied phenomenon of "melatonin suppression" by light [572, 573].

From these experiments in rodents, one would predict that the magnitude of CREB-dependent signaling would affect the rate of melatonin synthesis, and therefore the degree of melatonin suppression by light. To test this hypothesis, 16 additional subjects were recruited; fibroblasts were cultivated from each, and infected with CREB reporter. From the same subjects, the degree of melatonin suppression in response to an evening light pulse was also measured by half-hourly sampling of salivary melatonin before, during, and after light administration. As predicted, an inverse correlation was observed between the degree of melatonin suppression and the magnitude of CREB signaling in fibroblasts, reflecting the dependence of melatonin synthesis upon CREB (Figure 5A,B). Thus, signal profiling in fibroblasts can be relevant to various complex responses in other tissues that are impossible to access directly. (Note that absolute levels of melatonin are critically dependent upon the size and calcifications of the pineal gland itself, so no correlations were observed between CREB and peak melatonin levels in these subjects.)

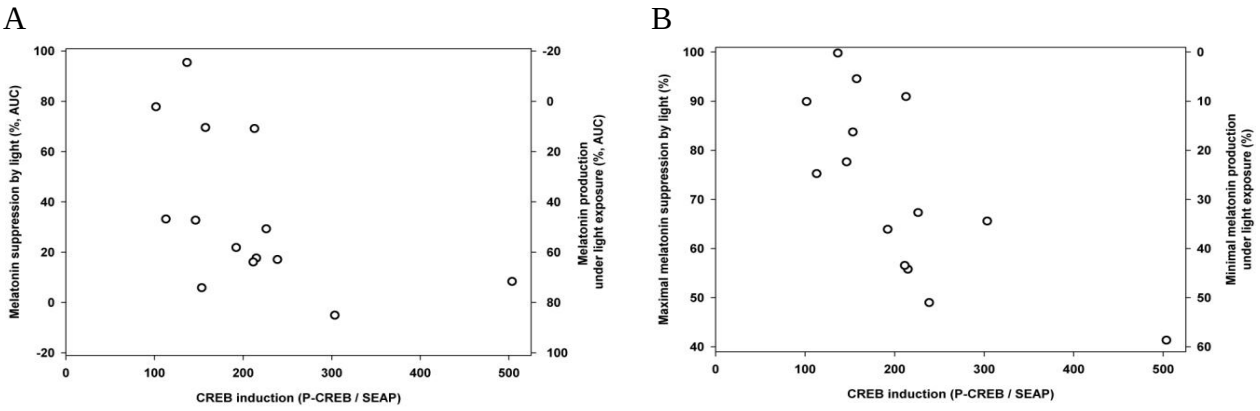


Figure 5 CREB expression in fibroblasts predicts melatonin suppression and production in human subjects. Correlations between melatonin suppression (left y axis) or melatonin production under full spectrum light exposure (right y axis) in human subjects is plotted compared to fibroblast CREB induction in response to forskolin (x axis). A) The area under the curve from the full spectrum light condition divided by het area under the curve from the dim

light condition is plotted against CREB induction (r = -0.666 p = 0.009). B) The minimal melatonin value from the full spectrum light condition divided by the maximal melatonin value from the dim light condition is plotted against CREB induction (r = -0.688 p = 0.007).

3.2.3 DISCUSSION

Trait-like variation in the expression of individual genes in a given tissue is well-known, and the invention of technologies to profile the expression of all RNAs in the transcriptome has enabled correlations between patterns of expression of specific gene sets with specific disease states, for example in different tumors [574]. Similar technologies have been applied to look at differences in gene expression that correlate with patient responses to different drugs [457], and comparable methods have been equally successful with the proteome (the sum of all proteins produced by the transcriptome) and the metabolome (the sum of all small molecules enzymatically created by the proteome) [575]. A major problem with such technologies is that effect sizes are typically quite small, probably because most genes possess regulatory elements that bind a host of different factors. For example, Zhang and colleagues used a chromatin immunoprecipitation (CHIP) as well as microarray approaches to identify CREB target genes in different human tissues [565]. The authors identified 4,084 putative CREB target genes. Nevertheless, only 100 or <2% were activated in response to forskolin, a strong stimulant of cAMP signaling.

In this study, we have used GAL4-based chimeric transcription factors to create a series of reporters that represent relatively clean, optimized promoters to detect activation of a single pathway, and delivered them into unmodified human primary cells via lentiviral vectors auto-normalized to eliminate differences in transduction efficiency. From these reporters, we find surprisingly high inter-individual variations in pathway expression among even a small sample of young, healthy individuals. In retrospect, the large variations that we observe make sense, because the reporters that we use are endpoints of signaling cascades designed to amplify small differences in receptor occupancy into nearly binary transcriptional responses. The validity of the inter-individual differences that we observe is confirmed by correlation with smaller transcriptional responses genome-wide measured via microarray, but only considering the most induced target genes for a particular pathway. Therefore, we conclude that pathway profiling of primary cells could provide valuable information missed by conventional approaches.

One obvious application of such information would be to inter-individual differences in drug response. To demonstrate this, we have treated cells showing minimum and maximum induction of the CHOP signaling pathway with methyl methansesulfonate, which induces cytotoxic endoplasmic reticulum stress and DNA damage at high levels. Since the CHOP pathway promotes apoptosis, we reasoned that cells showing high induction would be more prone to cell death, and that was indeed the case.

Of course, in order for such a cellular method to be useful for actual personalized medicine, the differences in pathway response observable in fibroblasts must be able to be generalized to other tissues. Even if it is clear that signaling pathways in general are highly conserved, the question of how well inter-individual differences in gene expression are conserved from one tissue to another remains a matter of controversy [576, 577]. Therefore, we attempted to apply pathway profiling technology to a pathway well-dissected in rodents and well-studied but not mechanistically understood in humans. In rodents, synthesis of melatonin by the pineal gland is a well-characterized CREB-dependent process. In humans, the suppression of melatonin by light has attracted broad interest because of its possible role in seasonal depression [578, 579]. We show here that CREB induction in fibroblasts predicts melatonin suppression in the same subjects. Although this correlation is highly simplistic, it nevertheless works: since the effect of light is antagonistic to CREB-dependent synthesis, one would anticipate a negative correlation between CREB pathway induction and melatonin suppression, and that is exactly what is observed.

Many recent studies showed that the use and easy access of peripheral cells could be a valuable tool to study not only various aspects of disease connected with alterations in signal transduction pathways, but peripheral cells can also serve as a powerful model in the drug development and screening processes [580, 581]. Moreover, a rising evidence of a wide human inter-individual variability in a response to standard doses of drug therapy suggests that the cell based systems could be very useful in the assessment of the drug action, toxicity, or efficacy [582]. Recent papers have also highlighted that skin fibroblasts can even be de- and re-differentiated into different cell types such as cardiomyocytes and neurons to predict disease phenotypes [583-585]. Our data shows that the inherent conservation of basic signaling pathways can already allow simple fibroblasts to predict complex behavioral responses, and that wide

inter-individual differences in signaling pathway function in these cells could be highly useful to personalized medicine.

3.2.4 MATERIALS AND METHODS

Human subjects

Protocols used with human subjects in this study were approved by the ethical committees of the University of Groningen (NL) and the Karolinska Institute (SW). All subjects were university students (age 20-24), equally gender-distributed, with no regularly used medications except oral contraceptives.

Tissue isolation and culture

As described previously[563], human primary fibroblasts were isolated from two skin biopsies from 10 young healthy subjects by 4 - 7h digestion of the tissues in 2ml of DMEM containing 10%FBS, 1% Penicillin/Streptomycin, 1% gentamycin, 0.325 W.U./ml Liberase Blendzyme3. After the isolation, human primary fibroblasts were kept in DMEM/ high glucose medium supplemented by 20% FBS, 1% Penicillin/Streptomycin, 1% gentamycin and 2.5µg/ml Amphotericin B. Antifungal Amphotericin B was left out from the growing medium after one week.

Vector production

The cell-based reporter system used for the measurement of the transcriptional activation of CREB, Elk1 and CHOP signaling is based upon the Stratagene In Vitro Path Detect system. This system features a reporter construct coding for firefly luciferase downstream of a synthetic promoter which is joined to five tandem repeats of GAL4 binding elements. A transcriptional activator construct containing coding sequences for the activation domains of CREB, Elk1 or CHOP fused with the DNA binding domain of yeast transactivator GAL4 is separately expressed from the CMV promoter. Lentiviral derivates of this reporter system were produced by the restriction cloning of the coding sequences of activator plasmids (pFA2 - CREB, pFA2 - Elk1 and pFA2 - CHOP) and reporter plasmids (pFR-Luc) into the pENTR4 gateway entry vector (Invitrogen) between attL1 and attL2 sites. Ultimately, Gateway® LR Clonase® II reaction was used to recombine and transfer inserts from entry vectors into a lentiviral destination vector

containing either Hygromycin (pLDEST-CREB-Hygro, pLDEST-Elk1-Hygro, pLDEST-CHOP-Hygro) or Puromycin (pLDEST-FR-Luc-Puro) selection marker. In addition to the reporter and activator construct, a third virus consisting of a secreted alkaline phosphatase gene under control of the CMV promoter was also included. All viruses were produced and concentrated as described in Cepko C., 2001 [586].

Bioluminescence measurement of the transcriptional activation of different signaling pathways

Semiconfluent plates (2.5 -5 x10^5 cells in 35mm Petri dish) of primary fibroblasts initially transduced and selected for the lentiviral luciferase reporter construct carrying puromycin resistance (pLDEST-FRLuc-Puro) were additionally cotransfected with the activator (pLDEST-CREB-Hygro, pLDEST-Elk-1-Hygro or pLDEST-CHOP-Hygro) and normalizing lentivirus vector (pLDEST CMV-SEAP Hygro) without selection. Prior to bioluminescence measurement of the transcriptional activation, aliquots of the medium taken from the fully confluent samples were used to detect levels of secreted alkaline phosphatase by Phospha-Light™ Secreted Alkaline Phosphatase Reporter Gene Assay System (Cat. N. #T1015). Ultimately, DMEM medium high glucose, w/o phenol red, 10% KnockOut™ Serum Replacement, 1%penicilin/streptomycin, 1% Gentamycin, 0,1mM luciferin was used to measure basal levels of the light emission during 20 - 30 min. Consequently, pathway specific drug stimulus was added to the tested samples (5µM Forskoline for CREB activation, 100ng/ml PMA to induce Elk-1 and 10ng/ml MMS for CHOP) and the signal was recorded over the 24-48 hours.

Data analysis and normalization

A single experiment included samples measured in four replicates, from two biopsies. Each sample was normalized by dividing the activator activity (maximal photon count value of CREB, Elk-1 and CHOP induction) by the control reporter activity (photon counts corresponding to SEAP values). Replicate samples were then averaged and the same calculation was done for all tests. Results are expressed as average +/- standard error.

Protein purification and Western Blot Analysis

Proteins from uninduced and induced human primary fibroblasts samples (for CREB – Subject 1, 10; Elk-1 – Subject 10, 5; CHOP – 7, 8) were extracted at the time of the maximal

transcriptional activation (CREB – after 5h, Elk1 – after 8h, CHOP after 24h) according to the NaCl - Urea - NP-40 protocol previously described by Laverz and Shibler (1993). 25 µg of protein was resolved by 10% SDS-PAGE, transferred to Protran® Nitrocellulose membrane, rinsed in PBS-0.1% Tween , and incubated 1h at room temperature with either primary polyclonal antibody against Phospho – CREB. (1:50 dilution, Cell Signaling Technology, #9191S), or polyclonal antibody against Phospho-Elk-1(1:50 dillution, Cell Signaling Technology, #9181) IRDye 680 Goat anti-Rabbit IgG secondary antibody was used in dilution 1:10000, 1h, room temperature. Washed and dryied membranes were used to detect and analyse the signal intensities by using the Odyssey infrared imging system (Li-Cor).Anti – Actin, clone C4 Monoclonal Antibody (Cat. No. #MAB1501) was used as a loading control for each immunoblot.

RNA isolation, whole genome expression analysis

Total RNA from CREB (Subject 1,10), Elk-1 (Subject 10, 5) and CHOP (Subject 7,8) uninduced and induced fibroblast samples were extracted as described by Xie and Rothblum 1991. RNA quality and quantity was determined by Agilent 2100 Bioanalyser. Single color gene expression microarray analysis was performed by using Human Whole Genome OneArray™ (Phalanx Biotech Group, Belmont, CA, USA). For each sample three technical replicates were performed. The data from all microarrays was passed to Rosetta Biosoftware for analysis, and returned a single average expression value for each gene. For each subject, induced values were divided by uninduced, and then ordered by fold induction. Relative fold inductions were calculated by dividing fold induction for a given gene and subject by the same value for another subject. To calculate values for an overall pathway, a list of pathway-specific genes was taken from [565, 566] and NEXTBIO Y platform for CHOP. Within this set, the average fold induction of the 50 most induced genes was then used as a measure of the overall induction of the pathway.

Viability Determination

In vitro sensitivity of the human primary fibroblasts from CHOP extreme subjects (Subject 7, 8) to an alkylating agent Methyl Methanesulfonate (MMS) was assessed by the Trypan Blue Exclusion Test of Cell Viability [587]. $1.5x10^4$ cells were seeded into 48 - well microculture plates and kept for an additional day in the incubator at 37ºC and 5%CO_2. On day three,

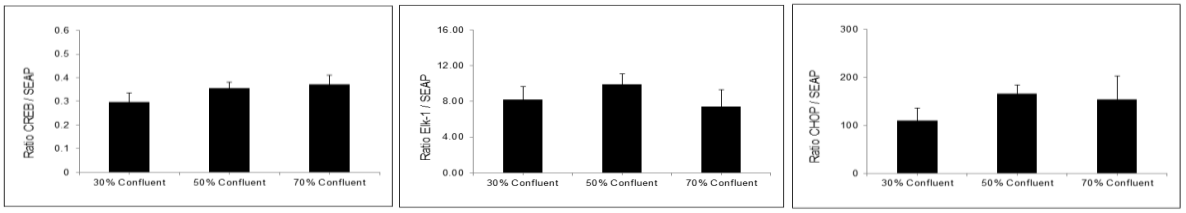
100ng/ml of MMS was added to the microculture plates and viability of fibroblasts cells was determined over the next four days. Measurement for each sample was done in six replicates and untreated cells were used as a control.

Measurement of melatonin suppression

The study consisted of 2 nights in the lab between June 19th and August 7nd 2010, the Netherlands. Each night was separated by a minimum of 1 and a maximum of 2 weeks. All subjects stayed in individual rooms. After 2 hours of dim light (< 5 lux) conditions, subjects stayed randomly in the dim light condition or in a full spectrum light condition (Philips TL-D36W/830 mounted on the ceiling, horizontal 627 ± 16 lux, vertical 164 ± 33 lux) from 23:00 till 7:00. Subjects stayed awake during the whole night and did computer (of which the screen was covered with a colour filter to block blue light transmission, E-colour +, 105 Orange; Rosco Laboratories Inc., U.K.) tests and questionnaires, which are not subject of this paper. They received hourly snacks of 100 Calories and 130 ml water. Each hour (from 21:30 till 6:30), using cotton swabs (Salivettes®, Sarstedt B.V. Etten-Leur, The Netherlands), saliva samples were collected for melatonin analysis, which was performed by radioimmunoassay (RK- DSM; Bühlmann Laboratories AG, Siemens Medical Solutions Diagnostics, Breda, The Netherlands).

Supplementary Figures:

A



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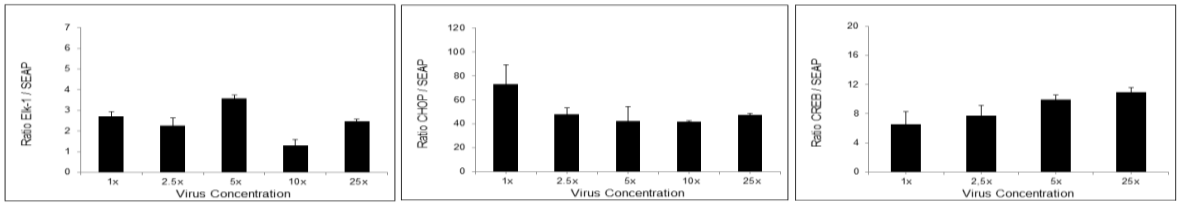

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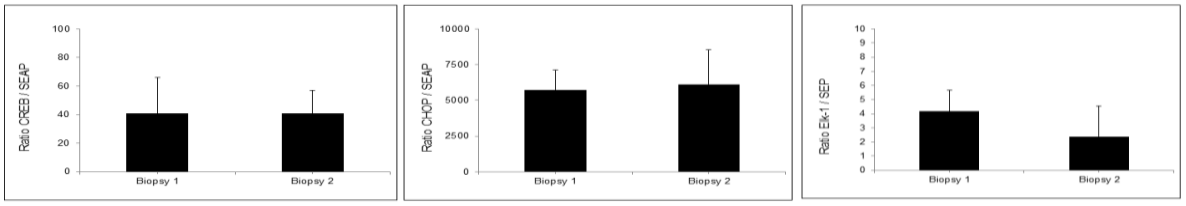

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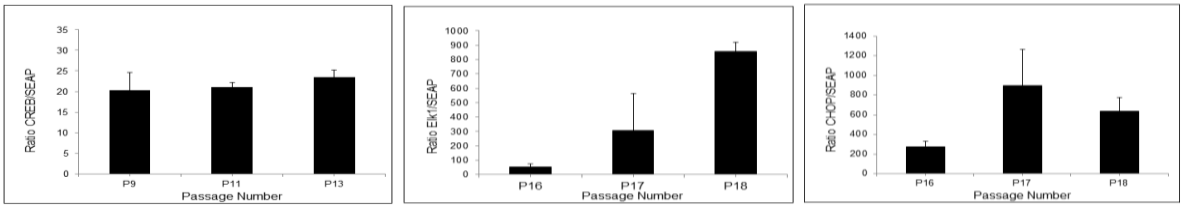
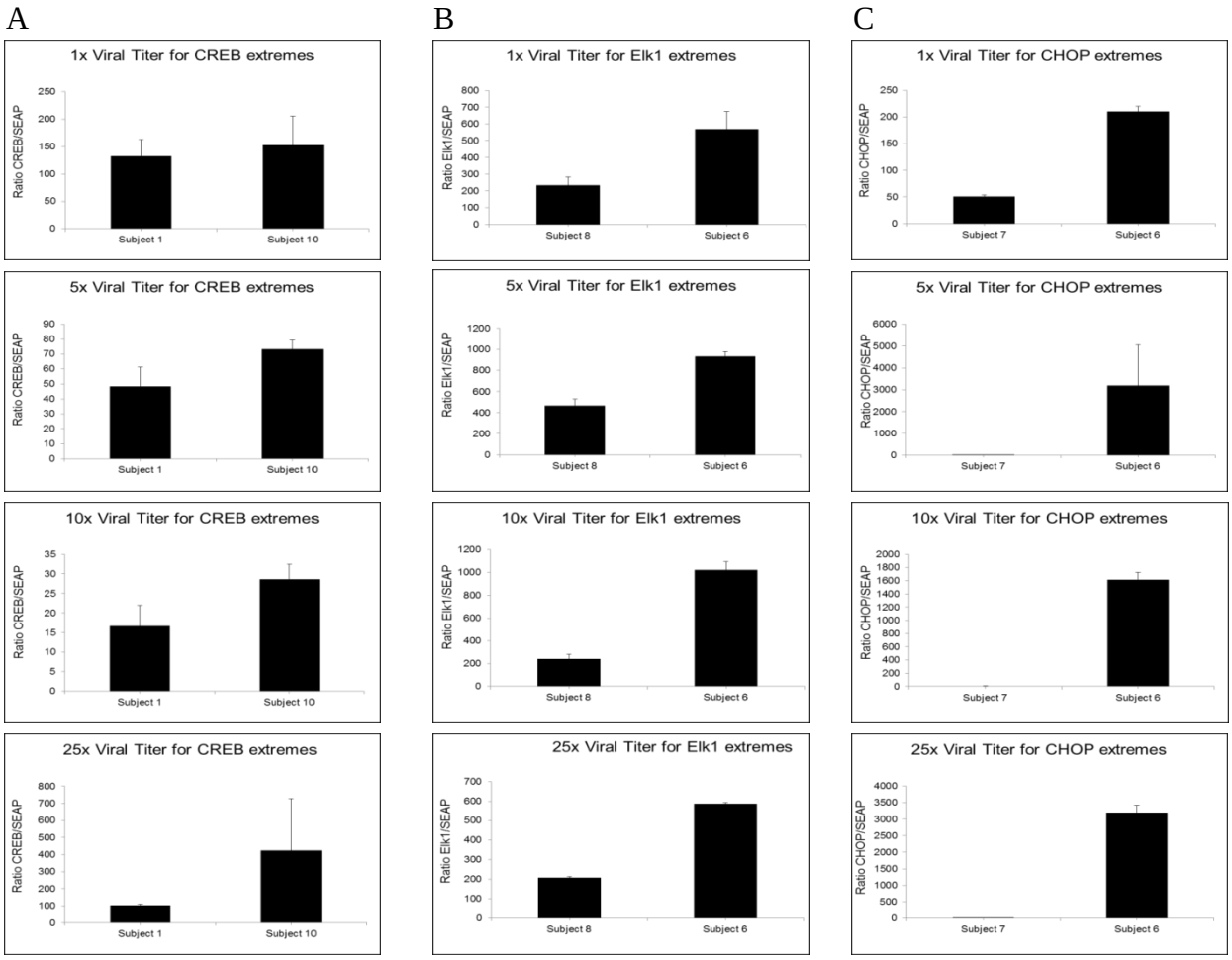
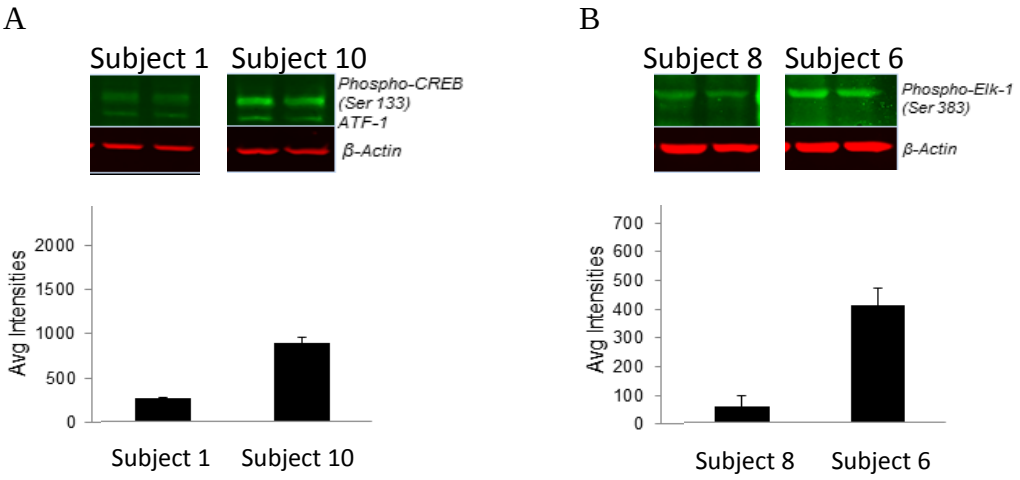


Supplementary Figure 1 Robustness of the lentiviral cell based system in response to experimental variation. Human cells from the same subject were infected with lentivectors for CREB (left panels), Elk1 (middle panels), and CHOP pathways (right panels). In each case, equivalent pharmacological inductions were performed under conditions of different **A**) Cell confluence, **B**) Viral Titer, **C**) Different biopsies from the same subject and **D**). Passage number. Data is plotted as the maximal induction observed in each experimental condition, divided by the expression of alkaline phosphatase in the same cells. Each sample represents the average +/- standard error for at least two replicates of two independent measurements. With the exception of passage number for ELK (expected because of its involvement in cell division and senescence), no significant differences were observed.



Supplementary Figure 2 Intersubject differences in pathway expression are independent of viral titer. For maximally and minimally inducing subjects for the CREB (left panels), Elk1 (middle panels), and CHOP pathways (right panels), as determined in Figure 2, human primary skin fibroblasts were transduced with different concentrations of the CREB, ELK, and CHOP lentiviral vectors. 1x Viral Titer represent unconcentrated filtered viral supernatant. 5x, 10x and 25x viral titers were concentrated by ultracentrifugation. Each sample shows the average +/- standard error from two replicates in two separate measurements.



Supplementary Figure 3 Different levels of endogenous phosphorylated CREB and phosphorylated Elk1 are observed in maximally and minimally inducing subjects. For subjects maximally and minimally inducing the CREB (A) and ELK pathway (B), each pathway was pharmacologically induced in fibroblasts using forskolin (A) or PMA (B). Whole cell extracts were prepared, and western blots were performed against phospho-CREB (A) and phospho-ELK (B) proteins. B-actin was used as a loading control. Top panels, raw blots; bottom panels, quantifications

3.3 Imaging of signal transduction pathways within the growing tumor

PREFACE: Self-sufficiency in growth signals, insensitivity to the growth inhibitory signals, evasion of apoptosis, limitless replicative potential, tissue invasion and metastases are all hallmarks of human tumorigenesis that have been found to be major signatures of deregulated cellular signal transduction cascades. Although these interconnected signaling events are being slowly elucidated, understanding the alterations that lead to cancer still remain a substantial challenge. Therefore, the same technologies that we have developed for the determination of natural inter-individual variation in the human cellular signaling could be equally suited to trace and study the behavior of signaling pathways within developing and progressing tumors. Since the cancer progression is a multilevel process that involves highly organized signal transduction pathways, better understanding the basis of the signal transduction alteration will certainly bring more insights into the variability of cancer pathogenesis among the human population as well as improve current approaches for cancer therapy.

http://www.iayork.com/MysteryRays/2008/09/14/immune-clearance-of-brain-cancer/

Imaging of signal transduction pathways within the growing tumor

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BACKGROUND

Tumor formation and progression occur via a broad range of defects that develop within and outside of the cancer cell. Defects in signaling pathways allow cancer to alter the normal programs of cellular proliferation, migration, transcription and even death. Changes in the surrounding tissues as well as in immune response potentiate the tumor to expand, form new blood vessels and spread into other organs. Therefore, different model system and various anti-cancer compounds have been developed to target different proteins that act in all of these pathways. However, rising evidence of inter-individual differences in the tumor development and progression as well as huge cancer heterogeneity complicates the development of successful cancer therapeutic strategies.

METHODS/RESULTS

To conduct *in vitro* and *in vivo* real-time bioluminescent measurement of signaling pathways in a mouse C51 allograft model, we employed our lentiviral cell-based system previously used for the profiling of natural inter-individual differences in human signaling. While the profiling of eight different signaling pathways (CREB, Elk1, cjun, CHOP, NFAT, NFκB, GAS, p53) *in vitro* revealed pathway-specific transcriptional activation, *in vivo* bioluminescence imaging of these signaling events showed mostly monotonic increase of the signal along with tumor development. To determine, whether the activity of signaling pathways can be modulated *in vivo*, a specific drug stimulus known to act on Elk1 and CHOP pathways was administered directly into a developed tumor. An increase in the bioluminescence signal was observed, demonstrating cellular responsiveness of the signaling cascade within the tumor to external stimulation.

CONCLUSIONS/SIGNIFICANCE

To reveal the behavior of major signal transduction pathways within the growing tumor in this study, we combined out robust lentiviral reporter system with a noninvasive longitudinal *in vivo*

imaging technique to reveal the behavior of major signal transduction pathways within the growing tumor. For the cascades examined, no significant temporal variations in signaling beyond those provoked by tumor size were observed. Nevertheless, we believe that this technology might help to shed more light into the action of various therapeutic compounds as well as improve the development of drug targeted technologies.

3.3.1 INTRODUCTION

The origin of cancer is a huge subject area with still too many unanswered questions. Although several lines of evidence suggest that human tumorigenesis is a multistep process in which different genetic and epigenetic alterations drive the progressive transformation of normal human cells into highly malignant derivatives, rapidly increasing evidence of human inter - individual variation in the cancer development and progression as well as differential responses of patients to the cancer treatment propose a rather more complicated picture of the cancer pathogenesis and therapy.

At the core of many regulatory systems of each living cell, signal transduction pathways play a dominant role. In general, these transductions encompass a multitude of interacting and cross-talking molecular cascades regulating the process of conversion of the external signals such as growth factors, neurotransmitters, cytokines or hormones to a specific cellular response such as gene expression, cell division or even cell death. It is also very well recognized that appropriate regulation of signaling pathways is essential for the maintenance of the overall cellular homeostasis, while on the other hand various alterations in this information flow can cause abnormal signal activation or inactivation that ultimately leads to the disease status such as cancer. Therefore, it is crucial to systematically analyze and understand gene expression signatures in the context of signaling pathways and uncover the mechanisms underlying pathogenic cellular phenotypes.

Many model systems, ranging from a simple *in vitro* cell culture to whole body *in vivo* molecular imaging have been established to decipher the crucial signaling components that play a role in human tumorigenesis. Although *in vitro* cell culture techniques are in principle valuable systems that complement more complex *in vivo* studies of signaling mechanisms underlying cancer development, molecular imaging is a particularly useful approach that allows the visualization of critical molecular signaling events in action in living subjects, in a noninvasive

and longitudinal manner. A variety of small animal imaging technologies like microcomputed tomography (CT), micropositron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI) as well as optical bioluminescence and fluorescence have been developed to monitor the cell biology of tumor development and progression [588]. Up to date, bioluminescence is the favorite technology for whole body optical imaging. Exceptionally high signal to noise levels, absence of background activity and a rapid turnover of the firefly luciferase protein with a relatively short half-life of~3h [589], predicts this system to be ideal to study kinetics and dynamics of gene expression within short time frames.

In the last few years of research, whole body imaging technology has become extremely useful for noninvasive and longitudinal monitoring of cell fate *in vivo*. This whole body bioluminescence imaging studies was not only successfully applied in studies of cancer cells has after their injection into living mice, but also for the monitoring of stem cells, to study the response of immune cells as well as tracking the rejection of allografts [590-594]. Moreover, this approach has been widely utilized to study oncogene regulation, genes involved in circadian clock rhythms, viral gene expression or genes involved in inflammation and various disease states [595-601]. In addition to the monitoring of endogenous gene expression, bioluminescence imaging (BI) approaches also found application in the visualization of efficacy of the transgene delivery and expression *in vivo* [602-604]. Besides gene expression studies at the transcriptional level, bioluminescence approaches also facilitate imaging of biological processes at the level of protein function and interaction. For example, Lehman *et al*. developed a fusion protein containing HIF-1α and firefly luciferase to study the stabilization of HIF-1α in the developing tumor [598]. Murine colon cancer cell line was transfected with this fusion reporter and subcutaneously injected into nude mice to form a C51 allograft model. I*n vivo* bioluminescence measurement revealed an interesting initial increase in HIF-1α level that was followed by the dramatic decrease when the tumor volume reached $1cm^3$. Apart from the detecting stability of given proteins, researchers also used BI to assess the activities in the degradation protein machinery. One example of such a work was published by Luker *et al*.in 2003. These authors generated a ubiquitin-luciferase reporter to determine the *in vivo* activity of 26S proteosome by assessing the degradation of the reporter [605].

Since many biological processes involve protein-protein interactions that are important for homeostasis of the overall cellular system, bioluminescence imaging found an application also in

this field of science. In this type of imaging, luciferase gene is split into two non-functional fragments, each of which is fused to one of the two studied proteins. Interacting proteins bring two fragments of luciferase close enough to re-form a fully functional luciferase. By using this approach, Luker *et al*. successfully imaged the activation and inhibition of chemokine receptor signaling in breast cancer *in vivo,* by detecting interactions between CXCR4 and β-arrestin. This study revealed a new imaging model to test CXCR signaling pathways and suggested the possibility to perform the screen for inhibitors in living animals [606].

Inspired by all these findings, a similar approach based on the whole animal bioluminescence imaging could be applied to study temporal changes in the activities of the major signal transduction pathways within the tumor microenvironment *in vivo*. Therefore, we utilized our robust lentiviral cell-based reporter system previously used in the study of human inter-individual differences, to trace the behavior profiles of eight signal transduction pathways in a mouse C51 allograft model. As cancer is an extremely complex and heterogeneous disease affecting many people over the world, better understanding of the molecular mechanisms that underlie the human cancer pathogenesis is necessary for the development of more efficient anti-cancer treatment, in which signaling pathways seems to play an attractive role.

3.3.2 RESULTS

3.3.2.1 Use of lentiviral reporters revealed pathway specific transcriptional activation in mouse colon carcinoma cell line

To determine the cellular transcriptional activity of eight different signal transduction pathways (CREB, Elk1, c-jun, CHOP, NFAT, NFκB, GAS and p53), we performed a lentiviral transduction of C51 cells. These cells have been incubated in the presence of lentiviral supernatant for at least twelve hours. For four days after the transduction, cells have been kept in the normal growth medium and puromycin / hygromycin selection scheme has been done to isolate positive transformants that stably express lentiviral construct coding particular signal transduction pathways. Subsequently, a real-time bioluminometry of drug induced signaling pathway activation was done in the presence of luciferin. Since some of our signal transduction pathway reporters are components of the MAP Kinase pathway (such as CREB and Elk1) that are easily inducible by various growth factors, cytokines etc., we needed to assure that the activation peaks for the respective signaling pathways obtained from our reporter assays are due to a response to the specific stimulus and not due to factors that might be present in the growth medium. Hence, to minimize an interfering effect, a protein rich fetal bovine serum was substituted for the defined serum replacement that was used for the following experiment. As shown in Figure 1A, even different concentrations of the serum replacement showed slight effects on the basal levels of some tested pathways. Therefore, rather lower (1-5%) but still sufficient concentrations have been used for all *in vitro* experiments. The overall raw profiles of the signaling pathways activation measurement are shown in Figure 1B. Beside the GAS and p53 signaling that seemed to be constitutively expressed within the C51 cell line, all other signaling pathways (CREB, Elk1, c-jun, CHOP, NFAT and NFkB) showed a robust drug induced activation with a specific kinetic pattern.

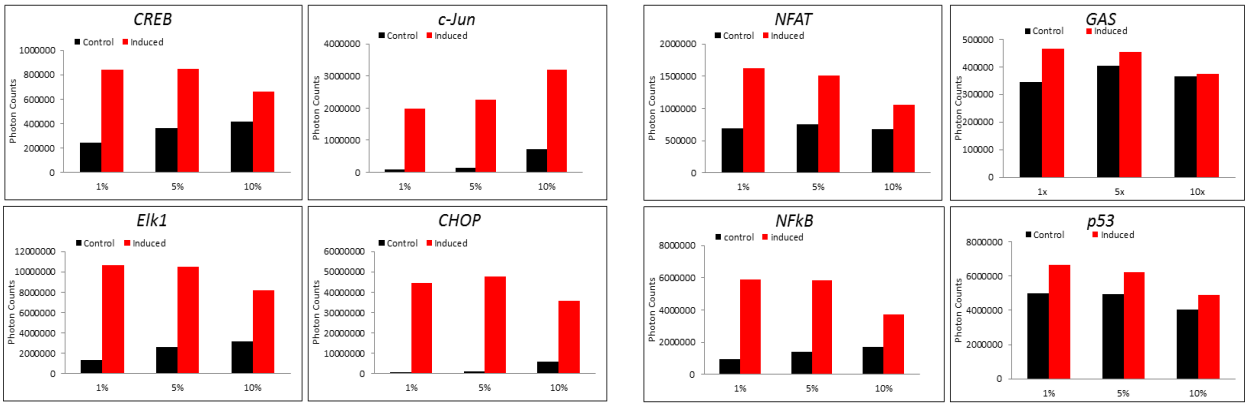

Figure 1A Effect of different concentrations of the serum replacement on the basal levels of tested signal transduction pathways. Black bars represent the basal levelsof pathways activation measured in the C51 cell line without the addition of the activator stimulus. Red bars represent pathway specific transcriptional activation in a response to a specific stimulus. For each set of measurement 1%, 5% and 10% serum replacement content was tested over 24-48hours. Reading time: 1min intervals. Drugs used for induction: **CREB** – Forskolin (5μM); **Elk1** - phorbol 12-myristate 13-acetate (PMA, 100ng/ml)); **c-JUN** – Dexamethasone (5μM); **CHOP** - methyl methanesulfonate (MMS, 10ng/ml); **NFAT** - phorbol 12-myristate 13-acetate (PMA)/calcimycin (0.5μM); **NFκB** - human tumor necrosis factor α (hTNFα, 50ng/ml), **GAS** - human tumor necrosis factor α (hTNFα, 50ng/ml);.**p53** - Actinomycin D (5nM).

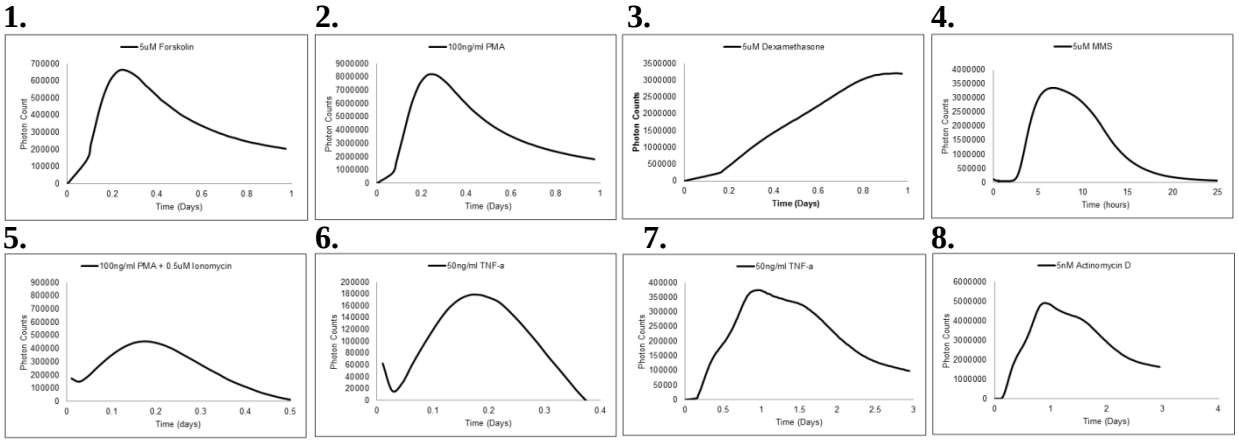

Figure 1B Pathway specific transcriptional activation in mouse colon adenocarcinoma cell line responds to specific drug stimulus. Raw bioluminescence profiles of the C51 cell line transduced with pathway specific reporters and then treated at time 0 with pathway specific activating compounds. Drugs used for induction: **1.CREB** – Forskolin (5μM); **2.Elk1** - phorbol 12-myristate 13-acetate (PMA, 100ng/ml)); **3.c-JUN** – Dexamethasone (5μM); **4.CHOP** - methyl methanesulfonate (MMS, 10ng/ml); **5.NFAT** - phorbol 12-myristate 13-acetate (PMA)/ calcimycin (0.5μM); **6. NFkB** - human tumor necrosis factor α (hTNFα, 50ng/ml), **7.GAS** - human tumor necrosis factor α (hTNFα, 50ng/ml); **8.p53** - Actinomycin D (5nM).

3.3.2.2 Longitudinal *in vivo* imaging of signaling pathways showed a monotonic increase of the signal within a growing tumor

To gain further insight into the complex and dynamic of signaling events, we performed *in vivo* real time bioluminescence imaging of chosen signaling pathways (Elk1, CHOP, NFAT and p53) in the growing C51 allograft tumors. The C51 cell line that has been stably transfected with lentiviral reporters and previously used for *in vitro* profiling of signaling pathways behavior, has been used for tumor inoculation in 8 to10 weeks' old BALB/C female nude mice. Five days after the inoculation, mice were anesthetized and intra peritoneal injection of luciferin was followed by a bioluminescence measurement performed in the light-tight chamber with a charge-coupled device imaging camera; IVIS 100, Xenogen. As showed in Figure 2 representing raw data of an actual measurement, CHOP, NFAT and p53 signaling pathways revealed only a monotonic increase of the signal within the growing tumor what could suggest that the tested pathways seem to be constitutively active within the *in vivo* environment of the tumor.

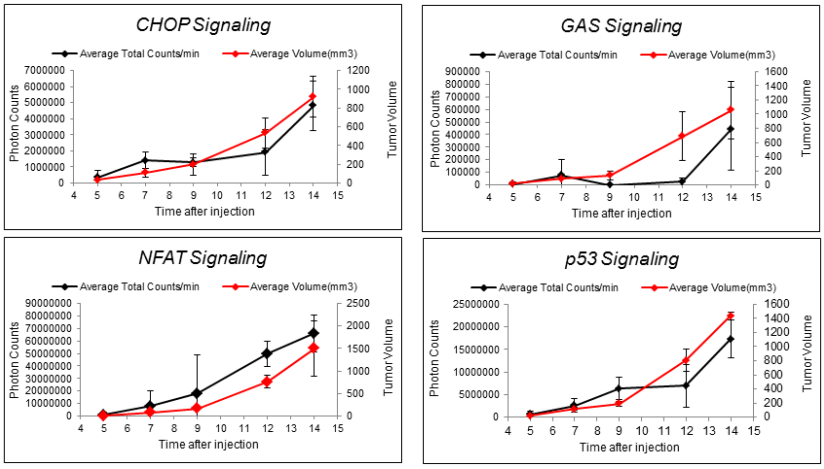

Figure 2 In vivo bioluminescence imaging of signaling pathway kinetics in C51 allograft tumors. In vivo bioluminescence imaging began on day 5 after the tumor inoculation. Altogether 5 data points for each pathway were measured. The black curve represents the average total photon counts/min; the red curve represents the average tumor volume; Error bars represent standard deviations of measurements in three mice per pathway (N=3).

3.3.2.3 Elk1 and CHOP pathways showed increase in their transcriptional activity upon a drug specific stimulation

Since many studies showed the involvement of Elk1 and CHOP transcription factor in the tumorigenesis and these two pathways also showed the robust *in vitro* induction pattern (Figure 1B) by using our lentiviral reporter system, in out further studies we decided to focus on these two signaling pathways. The C51 cell line that has been transduced with Elk1 and CHOP reporters was used for inoculation and *in vivo* monitoring of the transcriptional activity of these signaling pathways within a developing tumor. As shown in Figure 3, both signaling pathways revealed a monotonic increase in their expression pattern during nine days of the tumor growth. To test if these signal transduction pathways can respond to the specific drug stimulus also in *in vivo* environment, PMA (2.5µg of phorbol 12-myristate 13-acetate per mouse) and MMS (13.7µg of Methyl methanesulphonate per mouse) drug compounds, known to be acting on these pathways have been administered directly into the mouse tumor at the terminal ninth day of the measurement. An ultimate result revealed an interesting increase of the bioluminescence levels upon the drug stimulation what might suggest that the usage of longitudinal real-time bioluminescence measurement might in future reflect the drug evoked behavior of signal transduction pathways within the growing tumor in living mice.

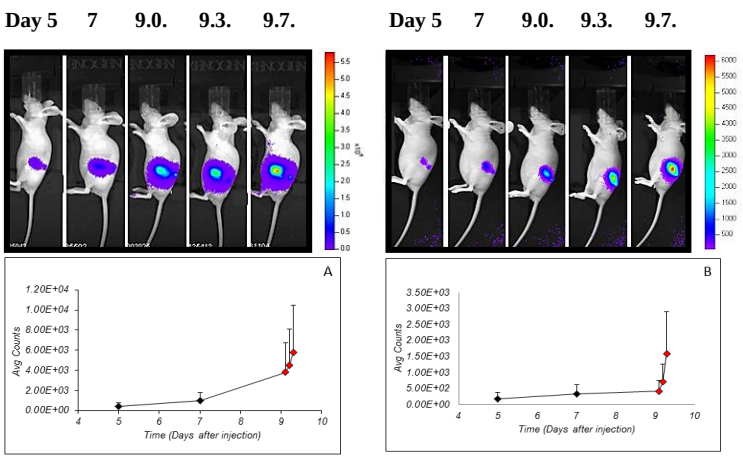

Figure 3 Monitoring the behavior of signaling pathways within the growing tumors. Upper panels: In vivo bioluminescence imaging of a) Elk1 signaling and b) CHOP signaling measured on a fifth, seventh and ninth day post injection. Three different time points were measured during the last ninth day. The 9.0. point refers to the time of the measurement before the injection of a specific pathway activator; the point 9.3. refers to the time of the measurement 3 hours after the injection of the activators and the point 9.7 refer to the time of the measurement 7 hours after the injection of the activators directly into the mouse tumors. Activators used for injection: Elk1 – PMA (2.5µg/mouse); CHOP – MMS (13.7µg/mouse). Lower panels: Charts representing bioluminescence measurement of both A) Elk1 and B)CHOP signaling. Red coloured points on the chart correspond to the time point measurements 9.0, 9.3 and9.7. Error bars represent the standard deviation (N=4 animals per pathway)

3.3.3 DISCUSSION

The induction of signaling pathways within cancer is often a result of genomic alterations such as mutations, translocation and copy number gains or losses, in crucial components of these pathways. Therefore, genomic technologies that are able to detect these aberrations represent important tools for identifying the so called "drug response biomarkers" of pathway specific signaling that are urgently needed for the rational selection of patient's cancer therapy. The activation of signaling pathways in cancer causes alterations in gene expression that are highly specific for the involved signaling pathway. Many studies have already demonstrated the potential of gene expression profiles for the analysis of oncogenic pathways as well as they helped to define the cancer subtypes, recurrence of disease and response to specific therapies [607-609]. However these DNA microarray-based gene expression signatures suffer by the fact that most of them reflect only a single point situation. Therefore, many various reporter systems and optical imaging technologies became more often used to reveal the underlying mechanisms of human tumorigenesis.

In this study, we combined the robust lentiviral cell-based reporter system with a long - term *in vivo* imaging technique with the aim to reveal the behavior of main signal transduction pathways within the developing tumor. A big advantage of this noninvasive imaging technique arises from a longitudinal and dynamic monitoring of cellular and molecular processes in the same subject. In our initial *in vitro* screen we determined which signaling pathways from the set of eight tested are able to respond to a specific drug stimulus. Apart from GAS and p53 signaling that seem to be insensitive to our drug stimulus and therefore they are constitutively active in C51 cell line, we obtained a pathway-specific kinetic pattern for all other tested signaling events. To reveal the kinetics and behavior of signaling pathways within the microenvironment of tumor *in vivo,* we injected a C51 reporter cell line into BALB/C nude mice. While five time point measurement didn't reveal any significant pattern for the majority of signal transduction pathways, *in vivo* administration of a specific drug compounds directly into the tumors modulated the transcriptional activity of Elk1 and CHOP pathway.

Although the longitudinal monitoring of the signal transduction pathways activity directly in the developing tumors seems to be a powerful tool that might bring more insights into the human cancer pathogenesis, we experienced significant variability of the signal distribution within the tested group of animals, during our measurements. To compensate for this variation in the signal

intensity that could be associated with changes in a tumor volume, blood absorption or tumor necrosis, the development of a normalizing system is required. Recent publication by Lehmann and her colleagues was focused on the role of tumor hypoxia and the hypoxia inducible factors in the growing cancer. In this study, the authors estimated the luciferase activity by a normalizing measurement of both HIF-1 luciferase and luciferase control tumors to the theoretical counts calculated for luciferase control tumors [598]. We attempted to apply this type of normalization on our existing dataset; however enormous discrepancy between the raw and normalized data was obtained (data not shown). Therefore, other kind of normalizing system, such as dual marker strategy possessing for example two distinguishable luciferase constructs, could be more suitable for the correction of possible inter-tumor variances [610].

Many recent studies revealed the relationship between cancer pathogenesis, signaling pathways and circadian clocks. As it is generally known, circadian rhythms rule many aspects of human physiology and therefore it is not so surprising that any genetic or epigenetic alterations might lead to the development of various disease states as for example cancer. For instance, from all known core clock genes, *Per2* has been shown to have an important role in tumor progression [611, 612] and its deregulation has been found in many types of human cancers [411, 412]. Moreover, genetic studies revealed that mice with dysfunctional circadian rhythms are more susceptible to many kinds of cancer development [613, 614]. In addition to this discovery, overexpression studies done on *mPer2* gene showed an inhibition pattern of cancer cell growth in both cellular *in vitro* systems and a xenograft mouse model. Further studies of this phenomenon exhibited possible mechanism where C-erB-2 and p53 were suggested to act as downstream players for *hPer2* in the course of tumor progression [402, 615-617]. Taken together, all these studies suggest a crucial role of circadian clocks in tumorigenesis. Therefore, it would be worth in the future to look at the kinetics of tumor's signal transduction pathways within a more dense time window, than is offered by current long-term optical technologies.

Determining the principal mechanisms that underlie cancer pathogenesis might offer the possibility for novel therapies. One of the therapeutic concepts that appear to have a big potential in the current cancer treatment is chronotherapy. Cancer chronotherapy - the timed use of chemotherapy that is based on circadian rhythms of individuals, certify the importance of understanding cellular dynamics to optimize therapy using the physiological drug doses as well as uses the differences among the cellular rhythms of normal and tumor cells [618-620].

Although it is still not completely employed in the clinical practice, modeling of cellular dynamics such as drug absorption, distribution and metabolism might be important in the identification of optimal, temporal windows for drug therapy. Thus, the specific timed drug administration seems to play an important role in various treatment strategies. So far, our initial results suggested only monotonic increase in the transcriptional activity of tested signaling pathways. At this point, we can only speculate what this result might mean for the therapy. Since, we didn't look closer at the circadian function of the C51 cell line within the context of tested signaling pathways; the obtained results might simply suggest that there is no importance of specific time for the treatment in the case of the pathways that we tested and the physiological doses of the drug compounds would have to be kept in the constant level. Nevertheless, it would be needed to determine the activity status of signal transduction pathways within denser time frame such as 24hours as well as we should consider the impact of circadian clock function, since some signaling pathways showed diurnal variation in their activity [621]. Moreover, it would be interesting to compare the kinetics of signaling events across different cancer cell lines. Although signal transduction pathways are highly conserved among various cell types, it is possible that we would find differences in the signaling behavior. As cellular behavior is regulated by the gene activity it would be logical to assume that differences in invasiveness or aggressiveness of tumor cells could be inferred from differences in gene expression.

Lastly, comparison of the expression profiles of tumor activated / inactivated signaling pathways obtained from different patients as well as a detailed screen of possibly differential treatment responses among these human individuals would also add some more light into the therapeutic approaches of cancer. Our *in vivo* activation experiment suggests that activities of signal transduction pathways traced within the developing tumor can be modulated by drug compounds known to be acting upon these pathways. Therefore, we can imagine that various drug toxicity and efficacy experiments could be initially performed in a simple *in vitro* screen and then followed by a more sophisticated *in vivo* longitudinal imaging of the target signaling pathways directly in the developing cancer.

3.3.4 MATERIAL AND METHODS

Vector production

The cell-based reporter system used for the measurement of the transcriptional activation of all eight signaling pathways is based on the Stratagene In Vitro Path Detect system. This system is described in detail in chapter 3.4 Materials and Methods.

In vitro Bioluminescence measurement of the transcriptional activation of different signaling pathways

Semiconfluent plates (2.5 -5 x10^5 cells in 35mm Petri dish) of C51mouse colon adenocarconoma cells which were initially transduced and selected for the lentiviral luciferase reporter construct carrying puromycin resistance (pLDEST-FRLuc-Puro) were additionally cotransfected with the activator (pLDEST-CREB-Hygro, pLDEST-Elk-1-Hygro or pLDEST-c-Jun-Hygro, pLDEST-CHOP-Hygro, pLDEST-NFAT Luc-Hygro, pLDEST-NF-κB Luc-Hygro pLDEST-GAS Luc-Hygro pLDEST-p53 Luc-Hygro) and selected. Ultimately, DMEM medium high glucose, w/o phenol red, 5% KnockOut™ Serum Replacement, 1%penicilin/streptomycin, 1% Gentamycin, 0,1mM luciferin was used to measure basal levels of the light emission during 20 - 30 min. Consequently, pathway specific drug stimulus was added to the tested samples: CREB – Forskolin (5μM); Elk1 - phorbol 12-myristate 13-acetate (PMA, 100ng/ml)); c-JUN – Dexamethasone (5μM); CHOP - methyl methanesulfonate (MMS, 10ng/ml); NFAT - phorbol 12-myristate 13-acetate (PMA)/ calcimycin (0.5μM); NFkB - human tumor necrosis factor α (hTNFα, 50ng/ml), GAS - human tumor necrosis factor α (hTNFα, 50ng/ml); p53 - Actinomycin D (5nM). The signal was recorded over the 24-48 hours.

Tumor inoculation

All the experiments were carried out according to the laws and regulations of the Canton of Zurich (Licence 16/ 2011 Steven Brown). 8 ~ 10 weeks old BALB/C female nude mice (Janvier, France) were gas anesthetized by 1.5 ~ 2% isoflurane. 1 × 10^6 C51 cells stably transduced with the lentiviral reporters were injected subcutaneously on the left flank of the mouse. We transferred the mice back to the cage after injection, waited till them totally recovered from isoflurane and made sure they did not suffer from the injection. The mice were sacrificed after 15

days of tumor inoculation, when the tumor sizes reached about 2 cm^3. The tumor volume was calculated by the formula: tumor volume= (length×$width^2$)/2.

Bioluminescence imaging of signaling pathways behavior in growing tumors

The imaging of the transcriptional activity of Elk1 and CHOP signaling pathways has been done as previously described by Lehman et al., 2009. Briefly, mice were anesthetized by using 3% isoflurane and oxygen as a carrer gas. Each mouse was i.p. injected with 100µl of luciferin in PBS (15 mg/mL; Caliper Life Sciences). 10 minutes later, the animals were placed in a light – tight chamber consisting charge-coupled device imaging camera, IVIS 100; Xenogen. Bioluminescence measurement was done in 5 and 300 seconds intervals. Collected images have been analyzed with Living Image software (Xenogen) and IGOR image analysis software (Xenogen). Total photon counts were determined by drawing a region of interest ROI around the peak of photon emission. The border of a ROI was formed by those pixels whose signal intensity was 5% of the maximal signal in the ROI. To correct for the loss of signal associated with bigger tumor volumes we normalized total counts from those tumors to pcDNA3.1-luciferase control tumor counts.

CHAPTER 4

General Discussion and Perspectives

The regulation of gene expression is one of the most essential cellular functions that defines and maintains cell type specificity, shapes very complex profiles in health and disease and it is likely that a significant portion of the genetic signal is associated with the phenotypic variation. While the dissection of these genetic signatures at the level of whole organism might be quite demanding, genetic variants at the cellular level can be easily interpretable. In the last decade, a significant portion of the scientific community has been attracted by the genetic diversity that explains variation of gene expression and many studies have been devoted to characterize its genetic architecture [472, 473, 622]. Throughout my PhD studies I have been exploring aspects of natural gene expression variation in circadian as well as other major non-circadian signal transduction pathways. To decipher the extent of human signaling variation, I have developed a novel high-throughput viral system that is capable of measuring differences in the output of these transduction cascades among primary fibroblasts derived from different human individuals. Furthermore, I showed that this robust system can be utilized to trace the behavior of signaling pathways within the developing cancer. The following sections summarize the findings of these three studies and discuss other relevant advances and pressing issues in the field.

4.1 Individuality of human circadian behavior

Circadian clocks are biological signaling networks that exhibit a period of ~24 hours under constant environmental condition. They have been identified in a wide range of organisms, from cyanobacteria to mammals and it is widely believed that many aspects of human physiology, mood and cognition are regulated by this highly conserved signaling phenomenon [623]. Multiple studies focused on circadian biology revealed that in addition to a “master circadian oscillator” located in the suprachiasmatic nucleus (SCN) of the brain, nearly all mammalian peripheral tissues possess circadian clocks [149, 167, 624]. Moreover, peripheral clocks appear to have a similar molecular set up as the clocks orchestrating in SCN neurons. Thus, in both SCN

and peripheral cells, the circadian rhythms are thought to be based on delayed transcriptional/translational feedback loops [625]. In addition to the various tissues explant studies, Balsalobre *et al.* in 1998 demonstrated that even immortalized Rat-1 fibroblasts that had been kept in the culture for decades possess functional and robust circadian oscillators. Since then, circadian gene expression has been demonstrated in a broad range of cultured cell lines such as primary fibroblasts, murine NIH-3T3 fibroblasts, human U2OS osteosarcoma cells and various types of peripheral mononuclear blood cells [149, 287, 392, 527, 626].

The interest in the genetic basis of individual differences via the research of the human circadian clock formed the initial phase of my PhD studies. Humans show large inter-individual differences in organizing their behavior within the 24-hours day and several studies showed that this diurnal preference is highly heritable [512-515]. Diurnal preferences or so called chronotypes are associated with the differences in the circadian clock and the genetic basis of this phenotype has been well established in animals. However, the genetic architecture that underlies the distinct circadian organization in humans still remains unknown. Although several genetic approaches based upon the linkage and association studies have been used to define a causative single nucleotide polymorphism of various human pathological chronotypes [516, 518, 519, 522], only a few reports about the genetic basis of human natural diurnal variation have been published [339, 517].

Both because measuring human circadian function via behavior is a laborious process, and in order to gain mechanistic insight into human circadian variations, I set out to develop a high-throughput way to measure circadian clock properties (such as period length, phase and amplitude) in human peripheral tissues. Recent technological advances in the molecular biology have afforded the opportunity to monitor eukaryotic gene expression selectively and in real-time in living cells. Among a variety of available “reporter genes”, high sensitivity, excellent dynamic range, and low toxicity has made luciferase the most commonly used bioluminescent reporter in the analysis of eukaryotic transcriptional regulation [627-629]. To deliver such reporters, several chemical, physical as well as viral technologies have been developed to study gene functions and protein expression in the context of a cell. Although both, chemical and physical transfection technologies have been used in various studies [630-635], extensive optimization is very often required to balance transfection efficiency and cell viability. Moreover, these methods allow only short-term studies of transient gene expression and therefore would be unsuitable for my studies.

Due to these obstacles, the use of viruses as delivery vectors has been explored as an alternative method. Virus based vectors have several remarkable advantages. Apart from the fact that they can rapidly infect a broad range of human cells and can achieve high levels of gene transfer, they can also accommodate relatively large segments of DNA. For example, Adenovirus based vectors have impressive packaging capacity up to 7.5kb, can transduce nonproliferating cell and are relatively easy to manipulate using recombinant DNA techniques. Other vectors of interest include adeno-associated virus, herpes simplex virus, retroviruses and lentiviruses. Lentiviruses (e.g. HIV-1) are of particular interest because they can integrate into the host cell genome to allow stable and long-term transgene expression as well as they can infect quiescent cell [636]. In my study devoted to "Inter-individual differences in human circadian rhythmicity", I employed various transient as well as viral technologies to deliver circadian reporter construct into human umbilical cord fibroblasts (UCFs) and lymphoblastoid cell lines (LCLs). Since the cultures of human primary cell as well as LCLs are quite refractory to transient transfection technologies, lentiviral delivery system seemed a logical choice [535]. Furthermore, this reporter system already proved to be useful in several studies of human circadian properties [287-289]. As described in Chapter 3.1., to my big surprise, lentivirus mediated gene delivery revealed only circadian rhythms of low amplitude in UCFs and very poor transfectability with no circadian gene expression in LCLs. Moreover, Adenoviral, Herpes Simplex Virus amplicon vector-mediated gene transfer as well as electroporation of an Epstein-Barr Virus-based vector containing the circadian reporter construct also showed no circadian gene expression in LCLs. To verify these unexpected results, the levels of mRNA for all circadian core clock genes in an around a clock experiment were determined. However, as mentioned previously, the result of qPCR showed only random, inconsistent circadian gene expression of low amplitude in both UCFs and LCLs (Chapter 3.1.). Since all tested viral based technologies are currently used as the most powerful approaches to the study the gene expression in various mammalian cells, it is unlikely that other packaging and delivery methods (e.g. adeno-associated viral based system), would improve the transfection rates of LCLs. Moreover, the levels of mRNA of the circadian clock genes revealed also only irregular pattern, suggesting more and more suggest a hypothesis of strong down-regulation or even complete abolishment of core clock gene expression in LCLs.

Lymphoblastoid cell lines are generated by Epstein - Barr virus (EBV) transformation of the B-lymphocytes from the peripheral blood lymphocyte population [637, 638]. Although several

previous studies showed that primary B-lymphocytes as well as other mononuclear blood cells possess circadian gene expression [529, 534], until now there is no evidence of robust circadian gene oscillations in LCLs. Epstein-Barr virus is a member of the herpesvirus family that has been associated with various human tumors such as Burkitt and Hodgkin lymphomas, B- and T-cell lymphomas, as well as with nasopharyngeal and gastric carcinomas. In LCLs, EBV expresses six nuclear antigen proteins, two latent infection membrane proteins, two small RNAs and BamHI A rightward transcripts that interact with or exhibit homology to wide variety of antiapoptotic molecules, cytokines and signal transducers and therefore promote EBV infection, immortalization and transformation [639]. From all, two Epstein Barr nuclear antigen proteins, EBNA3A and EBNA3C have been found to have essential roles in EBV immortalization of B cells. Although the complete mechanism is not yet fully understood, it was demonstrated that these two factors are important for maintaining LCLs growth by repressing CDKN2A $p16^{INK4A}$ and $p14^{ARF}$ expression [640]. CDKN2A $p16^{INK4A}$, cyclin dependent kinase inhibitor 2A is a tumor suppressor protein that is encoded by the *CDKN2A* gene. $p14^{ARF}$ is an alternate reading frame product of the *CDKN2A* locus. Both p16INK4A and p14ARF are involved in the regulation of cell cycle and their disruption lead to elevation of mdm2 and therefore loss of p53 function and cell cycle control. My initial hypothesis was that immortalization of human B cells might lead to the disruption of circadian clock gene expression in LCLs. Although immortalization of LCLs is not exactly a 'cancer type transformation', one might only speculate about the impact of accelerated proliferation of B cells [537] on circadian clock gene function. For example, it was demonstrated that circadian clocks regulate the expression of many genes involved in cell cycle. However this interaction appears to be reciprocal. In breast cancer the expression of *Per* genes is deregulated and many tumor cells have lost circadian rhythmicity as well as daytime dependent cell cycle proliferation [114, 613, 641]. One can imagine that by an EBV transformation, suppression of important cell cycle genes might lead to deregulation of circadian clock gene expression in LCLs. However, a set of several experiments would have to be done to prove this hypothesis.

Other possibility that might explain the lack of circadian clock gene expression in LCLs could be built on the knowledge that mitogen-activated protein kinase (MAPK) pathway is constitutively phosphorylated in most LCLs [642]. As it is generally known, that circadian synchronization involves photic stimulation of the SCN via the retinohypothalamic tract and

glutamatergic dependent signaling pathway through which immediate-early genes and clock genes are expressed in the SCN [643-645]. Circadian photic signaling also involves MAPK family [117, 391, 646, 647] from which phospho-ERK1/2 exhibit rhythmicity in the SCN. Other than light, variety of signals such as PACAP, NGF or GRP modulate ERK activity that in turn controls various downstream pathways [17, 648-650]. Studies done in the SCN showed that constitutive activation of MAPKinase completely blocked the responses of the SCN to phase shifting light pulses *in vivo* [651]. Moreover, MAPK pathway phosphorylates a basic helix-loop-helix-PAS transcription factor BMAL1 at multiple sites and BMAL1-CLOCK induced transcription is also inhibited by expression of a constitutive active form of MAPK in HEK293 cells [652]. Altogether, these findings suggest that constitutive expression of MAPK pathway might be responsible for a disrupted synchronization or can directly inhibit transcriptional machinery of circadian core clock genes. MAPK kinase in LCLs has been shown to be constitutively active. This constitutive activation might be responsible for the ineffective synchronization of circadian phases among the lymphoblastoid cell, which in turn result in inability to determine circadian rhythms via real time bioluminescence measurement as well as at the level of mRNA. On the other hand as discussed previously, constitutive phosphorylation of MAPK can completely abolish the circadian rhythms of LCLs that are based on the coordinated function of transcriptional/translational feedback loops. To distinguish between these two possibilities, I would suggest to initially screen for the MAPK activation status in LCLs. If constitutive activation would be confirmed, inhibition experiments that according to my hypothesis and published literature should restore the circadian rhythmicity in LCLs would be done.

Since I wasn't able to see the robust circadian clock gene expression in LCLs, this cell line became completely unsuitable for my studies of the genetic origin that underlies variation in the human circadian behavior. Moreover, none of the umbilical cord fibroblasts that have been tested along with the LCLs showed circadian gene oscillations. Although in this case, weak circadian oscillations during the *in vitro* measurement have been observed, only irregular circadian gene expression was found at the level of mRNA. Dampening or low amplitude of circadian rhythms might be explained by loss of synchrony among these cells. Other synchronizing agents beside dexamethasone and serum would maybe help to improve the circadian rhythmicity in UCFs, if any exist within these cell lines.

Although lymphoblastoid cell lines as well as umbilical cord fibroblasts are available as reasonably large cellular libraries, they failed to be utilized for my study due to their lack of robust circadian rhythmicity. To accomplish this goal, other cell types, e.g. human primary skin fibroblasts could have been taken as an alternative. These cells possess autonomous and self-sustained circadian rhythms and it has been already shown that the lentiviral based reporter system is efficient to deliver circadian reporter construct into these cells. The Coriell Institute for Medical Research is one of the few providers of human cell repositories from which I attempt to order several primary fibroblast cell lines. The idea was to screen founders from existing human pedigrees which are commercially available. For promising pedigrees, resulting period lengths would be used as quantitative traits in standard linkage analyses in order to identify human loci whose polymorphism influences circadian period length. However, the detection of the circadian gene expression in these cell lines was equally unsuccessful because only one cell line of eight survived the shipment. Senescent cell lines came either with poor density or with a fungus or mycoplasma contamination. Due to these incredible problems, the only way to perform planned expression quantitative trait mapping studies would be to establish our own reasonably large library of human skin fibroblasts derived from independent young healthy individuals or from multiple generation human pedigrees. In addition, the genome-wide transcription information would have to be determined as well.

In spite of my unsuccess, I still believe that signaling outputs obtained from the human primary fibroblasts could in future serve as an excellent system to determine the genetic basis of human inter-individual differences not only in the circadian but also in other major signal transduction pathways.

4.2 Inter-individual variations in human cellular signaling

Several studies evolved efforts to map and characterize differences in human gene expression patterns associated with differences in cell and tissue type, physiological processes as well as disease. *In vitro* experiments explored many aspects of physiological control of human gene expression, including the identification of genes periodically expressed within the cell cycle [653], the dissection of signaling pathways [654] and response of human cell to various compounds [655]. Moreover, gene expression profiles began to help to understand the complex biological programs associated with health and diseases also *in vivo*. However, the nature and extent of variation in gene expression among healthy individuals is still a largely unexplored aspect of human biology. Novel approaches and investigations of human gene expression programs linked to a disease and their impact on the detection and diagnosis will depend on an understanding of their normal variation within and between individuals.

The system that I have developed provides a quantitative readout of signaling pathway function. Because it utilizes an ideal synthetic promoter and activator at the endpoint of each cascade to report this activity, it provides low-background, high-amplitude signals that detect inter-individual differences in signaling proportional to, but greater than those provided by microarray-measured gene expression in the same cells. As discussed in chapter 3.2., by using this system, I discovered notably large inter-individual differences in the peak expression of the three studied pathways: CREB, ELK1 and CHOP. Moreover, the existence of these variations was confirmed by genome-wide differences in transcription from promoters known to be activated by these pathways, as well as by differences in cellular toxicity and efficacy of pathway-specific drugs. Finally, to demonstrate the broader physiological relevance of these findings, I was able to relate reporter-detected inter-individual differences in human fibroblast CREB signaling with actual suppression of the hormone melatonin by light in the same human subjects, a process known to be CREB-dependent from rodent studies [60-62]. Thus, I demonstrated that my non-invasive cellular signaling profiling technology could be relevant to various complex responses in other tissues that are impossible to access. Taken together our data suggests that highly conserved inherent signal transduction pathways might allow simple fibroblasts to predict complex behavior responses and that large inter-individual differences in signaling function in these cells could be highly useful to for example personalized medicine.

Beside utilization of primary skin fibroblasts for my research, many recent studies revealed that easily accessible peripheral cells could be a valuable tool to study not only alterations in signaling pathways connected to various aspects of diseases but can also serve as a model in the drug development technologies [580, 581]. Moreover, several very recent papers also highlighted that skin fibroblasts can even be de-and re-differentiated into different cell types such as neurons, muscle cells or cardiomyocytes to predict and study various human disease phenotypes [583-585, 656, 657].

In addition to all of these interesting applications, I believe that with a suitably genotyped library of cell lines, one would be able to use our technology to identify genetic modifier loci responsible for the large inter-individual differences in the expression pattern of signal transduction pathways that we observed. Possible cellular library that certainly meet the requirements of genetic studies comprises umbilical cord fibroblasts. The power of this library was already determined by Dimas *et al.*, who identified unique and shared expression quantitative trail loci (eQTLs) among three different cell types derived from the umbilical cord [658]. Moreover, Borel *et al.* employed primary umbilical cord fibroblasts to reveal regulatory polymorphic variants strongly associated with miRNA expression levels [577]. Although these cell lines have been unsuitable for the determination of genetic modifiers that underlie variations in human circadian behavior (Chapter3.1.), the preliminary screen performed in this cell line has already shown a feasibility of this idea. Thus, eQTLs mapping strategies applied to this library have already allowed to identify individual regulatory elements implicated in gene expression variation. Coupling this method with the reporter gene technology that I have developed should offer considerable further contributions to our understanding of variations in human gene regulation.

4.2.1 Promise of *in vitro* gene expression profiling and eQTLs studies

Given the wide variations in the human genome, it is not surprising that for any individual tissue, variations have also been observed in the transcriptome (the sum of all RNAs transcribed from cellular DNA), the proteome (the sum of all proteins made from the RNAs), and the metabolome (the sum of all small molecules catalytically created by the proteins). Investigators have used the same quantitative approaches upon these substrates, for example correlating the abundance of a particular transcript with a particular cancer outcome [574], or a particular

metabolite with risk of diabetes or coronary disease [659], often in unexpected ways. For example, while trait-based approaches were certainly not required to link the abundance of saturated fats to risk of infarctus, the link between branched-chain amino acids and diabetes [660] only became apparent through diabetic metabolome analyses.

Recently, investigators have taken such analyses one step farther, treating the expression levels of transcripts, proteins, and metabolites among various individuals as quantitative traits in themselves, and relating them to particular genetic polymorphisms. Such "eQTLs" (expression quantitative trait loci) have been heralded to hold great promise for the elucidation of the causes of disease, based upon the assumption that polymorphisms in regulatory proteins, binding sites or genes are likely to be responsible for heritable variation in gene expression [661, 662]. eQTLs mapping has been already applied to a number of species ranging from yeast to humans as a powerful tool for determining various regulatory associations [663-671]. Nevertheless, due to limitations in sample size coupled with often very small effects, such studies have been notoriously variable and often yield quite large eQTL regions (up to several centimorgans) [672], leading some prominent investigators even to question their diagnostic potential [673]. For this reason, several strategies have been used to improve accuracy and narrow down the candidate causal gene regions, including traditional but frequently infeasible "fine mapping" [674-676], likelihood-based selection [677], and pathway-based approaches founded on the assumption that target gene expression correlates with the activity of other genes in the same pathway [678]. Such pathway-based approaches have been particularly useful for eQTL-based pharmacology, where drugs applied to cells are known to elicit responses in particular signaling cascades[679]. eQTL approaches in humans, though promising, are subject to a serious limitation that only a few tissues are readily available, such as blood and skin, except in certain clinical situations where biopsy is routine (in cancer, for example). Nevertheless, several recent eQTL studies revealed that the gene expression levels generated from various human samples including lymphoblastoid cell lines (LCLs) [473, 670], liver tissues [680] or primary lymphocytes [681] could be vigorously associated with *cis*-acting genetic variants. However, usage of immortalized versus primary cell lines for eQTL mapping has raised significant contradictions. While some studies highlighted cell type-specific effect, others found a significant amount of shared eQTLs across tissues [576, 577]. Other studies comparing the overlap of eQTLs found in LCLs and primary tissues revealed that some [682-684] but not all [685] eQTLs detected in LCLs can be

detected in even the same primary tissue. Hence, even though the same signaling pathways are widely conserved in all tissues, application of eQTL technologies to human populations effectively remains an important challenge.

4.2.2 Signaling pathway profiling vs. eQTL studies

So far, mapping of expression quantitative traits has focused upon determining genetic modifier loci for the expression of individual genes, as the word implies [686]. In these studies, we have rather taken the novel approach of focusing upon methodologies for determining quantitative assays for entire signal transduction pathways as functional units. This methodology has both advantages and disadvantages, as discussed above. Because the reporters that we have developed contain optimal synthetic transcription factor binding sites, fold-differences in transcription are typically higher in amplitude compared to effects at individual genes. Most importantly, however, inherent crosstalk is minimized. Treatment of cells with even the most specific pharmacological agents results in broad-scale transcriptional changes of enormous complexity. Our results showed that application of a pharmacological reagent specific for a given pathway – for example, forskolin to activate cAMP/CREB signaling, or dexamethasone to activate glucocorticoid signaling – activates first the expected pathway, and then multiple others. As a result, single-time point genome-wide measures of transcription fail to indicate which pathway was the primary target of the compound. In reverse, for a given tissue or cell line, only a small number of possible target genes is activated. For example, chromatin immunoprecipitation (ChIP) studies identified 10,447 promoters bound by the CREB protein upon activation of this pathway – already only a small subset of the 4,084 promoters with consensus binding sites. However, only 100 or <2% genes were actually detected as transcriptionally activated [565]. Thus, looking at the whole transcriptome, not only are many more genes activated by a single pathway than would be predicted directly, but also far fewer of the genes that could be predicted directly. Therefore, in looking broadly at signaling differences as quantitative traits, choice of different genes could yield hugely different outcomes. By using a simple optimized virally delivered promoter that is integrated into thousands of different sites in thousands of different cells, the methods that we have developed essentially bypasses some of this variability, providing a clear signal of high amplitude.

Even if signaling pathway profiling allows higher-amplitude quantitative traits which would translate directly into improved mapping of modifier loci, such analyses have one obvious drawback: pathways are composed of many components, and the reporter readouts that we use are essentially summed effects of modifications of each step. Therefore, while the method is diagnostic of defects or variations in a pathway, it provides no indication of *where* that variation occurs. For example, the most studied cAMP responsive element binding protein, CREB, is a transcription factor that is both a positive and a negative regulator of gene transcription that influences the expression of multiple genes. Activation of adenylyl cyclases, i.e. by forskolin, elevates the intracellular level of the cAMP, which in turns activates cAMP-dependent protein kinase A (PKA). Ultimately, PKA phosphorylates and therefore activates CREB and exactly this endpoint of cAMP signaling pathway we are able to measure. However, besides the adenylyl cyclases induction, membrane depolarization or calcium ion pore opening signals that elevate intracellular levels of calcium, activate calcium/calmodulin kinase, which also phosphorylate CREB. Moreover, the p90 ribosomal S6 kinases, RSK1 and RSK2, components of a growth and anti-growth signaling pathway, can also activate CREB. Additionally, CREB is phosphorylated by MSK1 and MSK2 that are kinases of the MEKK/MKK/p38 cellular stress pathway [687]. Thus, several signal transduction cascades converge at CREB. Therefore, it would be highly interesting to determine the distribution of human inter-individual variation based upon the activation of distinct signaling branches.

There are two principal repercussions of this complexity. First, since pathway profiling measures the summed activity of a given pathway, the results obtained are not necessarily applicable to any given gene regulated by that pathway. Transcriptome analyses have shown particular gene sets activated or repressed by each of the branched pathways above that regulates CREB signaling, and which are distinct or overlapping with the set of CREB-regulated genes. Thus, inter-individual variation in a particular branch might be better analyzed by survey of a known set of target genes, rather than an amalgamated index of pathway status. Secondly, the breadth of regulation of a single pathway means by definition that overall variation will be polygenic in nature. Until actual mapping studies are performed, it will remain unclear whether particular regulatory variants will be strong enough to rise above this genetic "noise", particularly when mapping variation in healthy individuals.

4.2.3 Pathway profiling as a phenotyping tool

In the preceding pages, I have outlined advantages and disadvantages of the methodologies that I have developed, mostly in view of gene discovery strategies. However, the system possesses great practical utility in a variety of ways. For example, our experiments revealed that inter-individual differences in pathway profiling directly correlate with cellular drug toxicity for the CHOP pathway. Therefore, the system possesses considerable versatility for drug screening. Indeed, such approaches have been used previously in many pharmacological studies. General toxicity tests have been carried out on many cell types such as fibroblasts, HeLa or hepatoma cells and parameters including viability, cytosolic enzyme release and cell growth are commonly used as end-points to measure toxicity [688-691]. Besides these cell types, very recent studies revealed that iPSCs also present interesting avenues of exploration for the drug discovery [581].

To fully realize the potential of this system, it would be useful to establish banks of fibroblasts from healthy and diseased subjects for a given disorder, or for patients responding to a particular drug vs. non-responders. In this way, it would be possible to estimate the utility of pathway profiling for endophenotyping of individual patient populations, as opposed to using it as a strictly cellular screening tool. For example, many mood disorders remain broadly classified on a symptomatic basis described over a century ago. Within these classifications, however, significant variations exist. For example, schizophrenia is marked by both so-called "positive" symptoms (psychosis, disordered thinking, etc.) and "negative" ones (social withdrawal, reduced motivation). Different patients are marked by different combinations of these symptoms. Moreover, the two sets of symptoms appear to have completely different functional correlates: whereas positive symptoms are in some way associated with brain dopamine levels, negative symptoms are correlated with actual structural changes in the brain [692]. Genetically, polymorphisms at the *Disc1* locus have been identified in a number of studies of schizophrenia as a possible source of risk. The DISC1 protein, in turn, participates in a number of signaling cascades, including the WNT pathway and the PI3/Akt pathway, which are conserved across all cell types [693]. Hence, specific dysfunction in signaling could conceivably be identified by pathway profiling at a cellular level, even in peripheral cell types. Such information could in turn suggest particular treatment options; currently a wide range of medications are prescribed on a symptomatic basis, with mixed success.

4.2.4 Pathway profiling for systemic differences

In my studies, I have focused upon inter-individual genetic differences in signaling pathway expression. However, the same system can be used in multiple different fashions. For example, one aspect of inter-individual genetics is the sum of genetic and epigenetic changes induced in cancer. Much as one can look at signal transduction signatures from cells from different individuals, one can look at similar properties in different tumor cells, both in culture and in xenograft models. This subject is treated separately as Section 4.3 below. Equally, however, cellular gene expression is influenced by external paracrine or endocrine signals, and differences in these can also be profiled. Our laboratory is currently using the technologies presented in this thesis in multiple pilot studies in this direction. For example, circadian reporters in primary fibroblasts were used to screen serum from older and younger individuals in order to identify serum factors contributing to circadian disturbances in elderly individuals [694]. More generally, it would be interesting to know how cellular signaling in general is affected in these individuals, both as a result of intrinsic genetic factors and circulating endocrine ones. For example, recently the chemokine CCL11 was identified as a circulating factor inhibiting neurogenesis in older mice [695]. In another project, our laboratory is using an array of primary fibroblast signaling pathway reporters to look at signaling pathways induced by endocrine factors in chronic fatigue syndrome. Since acute infection produces sickness behavior that is dependent upon the cytokine TNF-α [696], it might be expected that chronic fatigue – even if TNF-α-independent – might result from circulating cytokines that activate similar pathways, like NFκB.

4.3 Signal transduction pathways in cancer pathogenesis

Various signaling pathways regulate cell growth, differentiation, proliferation and death. Many common genetic aberrations in cancer involve various signaling components, either as activating mutations or loss of function of tumor suppressors. Since signals along the signal transduction cascades are typically propagated by different protein kinases many specific kinase inhibitors have already proven to be clinically successful. However, complete mechanisms underlying different cellular signaling pathways involved in human cancer pathogenesis still remain unclear.

Although high throughput 'omics' analysis of cancer patient gene expression resulted in the identification of key genes and signaling pathways that drive cancer progression [697] most of these studies reflect only single points of a multilevel and very complex process of cancer pathogenesis. Therefore various real-time and longitudinal approaches have been developed to crack the nature of cancers. Beside macroscopic imaging systems that provide physiological and anatomic information (e.g. CT/computed tomography, MRI/magnetic resonance imaging, US/ultrasound), molecular imaging technologies have found an application in clinical (PER/positron emission tomography, SPECT/single-photon emission computed tomography) or experimental (BLI/bioluminescence imaging, FRI/fluorescence-mediated tomography, MPM/multiphoton microscopy) use. In my study, I utilized long-term *in vivo* bioluminescence imaging technology coupled with the robust lentiviral cell-based system to uncover the signaling pathways behavior directly in a developing tumor. As discussed in detail in Chapter 3.3., *in vivo* bioluminescence measurement revealed only constitutively active profiles for the majority of tested signal transduction pathways although *in vitro* detection suggested that only two (GAS and p53 signaling) are constantly active and therefore insensitive to the pathway specific drug stimulus in C51 murine cancer line. This result is maybe not so surprising when one considers that the cell-cell and cell-environment interactions play important roles. One refreshing result of this study came in a form of modulation of signal transduction outputs upon *in vivo* drug application. Elk1 as well as CHOP pathway showed an increase in the transcriptional activity when pathway specific drug stimuli were administrated directly into the tumor.

The last few years of intense research has been focused on the development of the targeted therapies. In this approach specific drugs are directed at genetic defects in cellular signaling events that might occur in only small subpopulation of patients, thus proposing the concept and promise of personalized medicine. In oncology, distinct biomarkers are helping to characterize the signaling defects in selected patient subsets. Although identification of biomarkers is a key step for the targeted treatment, the so called pharmacodynamic biomarkers are important predictors of drug efficacy and toxicity that is very often altered by the presence of genetic polymorphisms in drug-metabolizing enzymes among human individuals [698]. Many of these biomarkers could be used to distinguish mechanistically distinct drug classes or can provide information about the expression activity of molecular targets in a response to a therapy. Undeniably, the methodology that I have used in my cancer study needs further optimizations

(such as development of the reporter system) as well as the measurements should be repeated to get higher impact of significance. Nevertheless, I believe that the two powerful technologies coupled in this study (characterization of the signaling pathways behavior was based upon the molecular imaging) will allow the development of biomarkers for signaling pathways and will provide better and importantly noninvasive measurement of drug effects. Consequently, they could be useful in preclinical and early clinical phases of the drug development. For instance, such biomarkers could serve to determine drug dose and identify the best duration and timing of a treatment or could identify possible toxicities and compare potencies of several drug candidates.

Oncogenesis is a complex process where not only accumulating genomic (e.g. gene dosage, allelic status, mutation in sequence) but also epigenomic defects (e.g. DNA methylation, histone modification, microRNA) significantly alters the cellular transcriptional programs. Various signaling pathways responsible for tumor progression lead to the upregulation of polycomb group protein, components of DNA methylation machinery. For example, overexpression experiments done in the context of Ras pathways demonstrated a requirement of these proteins in abnormal gene promoter DNA methylation [699]. Furthermore, it was revealed that overexpression of *c-myc* gene creates a signature of CpG island hypermethylation in culture and *in vivo* T-cell lymphoma models [700]. Similar observations have been published for Wnt/β-catenin signaling [701], ERK and NFκB as well as Notch signaling [702]. The fact that epigenetic changes are so extensive in different types of cancers, many therapeutic approaches directed to reverse gene silencing are under development. These compounds usually function as inhibitors of the DNA methyltransferase enzymes [703-705].

Although various drugs have already been developed, the majority of cell-line information about the abolishment of signaling pathways in cancer are based on molecular profiles of untreated cells. Real-time longitudinal imaging of signal transduction pathways within the developing tumors also offers a possibility to test different cancerostatic compounds directly *in vivo*. Moreover, since the rising amount of reports indicate the existence of not only natural inter-individual differences but also inter-individual variations in the therapy responses, it would be highly interesting to employ our system and look at the expression profiles of tumor associated pathways among the different patients suffering with the same type of cancer and to test different drug compounds. Last but not least, the dynamic variation of these signal transduction pathways

would have to be determined within a certain time frame such as 24 hours, since the circadian clocks and therefore specific time points of the drug administration seem to play an important role in many treatment strategies.

In conclusion, the principal achievement of my work is the development of a high-throughput technology for profiling of signaling pathways, both circadian and otherwise, in primary cells from different individuals. This technology provides a powerful tool for basic gene discovery using genome-wide mapping approaches in a variety of experimental contexts. It provides a way to analyze directly how signaling pathway function is influenced at a cellular level on the one hand by genetic factors, and on the other hand by circulating cytokines that could be genetically or environmentally regulated. The resulting information should be applicable to the study of many physiological disorders. Moreover, the same technologies could be directly applied to the phenotyping of human disease or drug response, providing a direct way to validate particular diagnoses or therapeutic approaches. For all of these applications, my contribution represents a promising beginning. In the years to come, future studies both in our laboratory and that of others will prove instrumental in translating these methodologies for purposes of clinical practice and scientific discovery.

4.4 Perspectives

The importance of natural gene expression variation to differences in human behavior and physiology is by now undisputed, and eQTL studies have shown not only that gene expression levels differ among individuals, but have also revealed unexpected and interesting aspects of gene regulation [670, 706]. The profiling technique that I have developed for the analysis of cellular signal transduction pathways could have a significant impact not only for basic research but as well for its application to medicine. In the area of basic research, identification of genetic modifier loci that might correlate with a variety of difficult-to-access behavioral phenotypes (daily behavior, memory consolidation, or mood) will bring more insights into the knowledge of natural human variability regulated by the complex operation of these pathways.

Apart from basic research, however, I believe that my methodology could be directly applicable to the fields of pharmacology and medicine. The last century has been devoted to discovery of new medical treatments of scores of human diseases. Nevertheless, most drugs do

not act equally upon all people. Increasing evidence of inter-individual differences in pharmaceutical drug treatment has greatly increased interest in the field of so-called "personalized medicine". Until now, two main factors are thought to be responsible for variability in drug toxicity and treatment efficacy: first is a matter of timing (e.g. chronopharmacology; timed treatment) and a second is the actual existence of inter-individual variation in human physiology (e.g. inherited metabolic differences, genetic polymorphism in drug metabolizing enzymes or target receptors, etc.) [456].

My initial results suggest that we are indeed able to see trait-like behavior (inter individual differences) in signal transduction pathways in peripheral cells. Since the behavior or expression pattern of these pathways is in fact a response to various pharmacological stimuli, I suppose that the signals obtained from our assay could be used equally as valuable biomarkers for individual therapeutic response. Thus, my method can possibly be used as a noninvasive and potentially high-throughput screening technology for many pharmaceutical compounds directly in human peripheral cells. Therefore, it would be highly interesting to look at possible differences in case and control individuals for many complex disorders, as well as on "*in vitro* drug treatment". For instance, many affective disorders, including depression, bipolar disorder, schizophrenia etc. are thought to be a result of various signal transduction pathways alterations. Identification of biomarkers for mood disorders that are capable to predict the response to the treatment is a highly challenging task. Until recently, different levels of neurotransmitters have been used as primary biological markers found in mood disorders. However, the discovery of various players within signal transduction pathways in neurotransmission has begun to redirect the focus of many scientists in a hunt for new biomarkers. Differences in for example signal transduction pathways endpoints as well as differential activation of target genes could play a crucial role in providing new treatment targets for a variety of diseases.

References

1. Garaulet, M., J.M. Ordovas, and J.A. Madrid, *The chronobiology, etiology and pathophysiology of obesity.* International Journal Of Obesity. **34**(12): p. 1667-1683.
2. Vandenpol, A.N., *The Hypothalamic Suprachiasmatic Nucleus Of Rat - Intrinsic Anatomy.* Journal Of Comparative Neurology, 1980. **191**(4): p. 661-702.
3. Moore, R.Y. and N.J. Lenn, *Retinohypothalamic Projection In Rat.* Journal Of Comparative Neurology, 1972. **146**(1): p. 1-&.
4. Stephan, F.K. and I. Zucker, *Circadian-Rhythms In Drinking Behavior And Locomotor Activity Of Rats Are Eliminated By Hypothalamic-Lesions.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 1972. **69**(6): p. 1583-&.
5. Moore, R.Y. and V.B. Eichler, *Loss Of A Circadian Adrenal Corticosterone Rhythm Following Suprachiasmatic Lesions In Rat.* Brain Research, 1972. **42**(1): p. 201-&.
6. Raisman, G. and K. Browngrant, *Suprachiasmatic Syndrome - Endocrine And Behavioral Abnormalities Following Lesions Of Suprachiasmatic Nuclei In Female Rat.* Proceedings Of The Royal Society Of London Series B-Biological Sciences, 1977. **198**(1132): p. 297-314.
7. Vandenpol, A.N. and T. Powley, *Fine-Grained Anatomical Analysis Of The Role Of The Rat Suprachiasmatic Nucleus In Circadian-Rhythms Of Feeding And Drinking.* Brain Research, 1979. **160**(2): p. 307-326.
8. Ralph, M.R., et al., *Transplanted Suprachiasmatic Nucleus Determines Circadian Period.* Science, 1990. **247**(4945): p. 975-978.
9. Inouye, S.T. and H. Kawamura, *Persistence Of Circadian Rhythmicity In A Mammalian Hypothalamic Island Containing The Suprachiasmatic Nucleus.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 1979. **76**(11): p. 5962-5966.
10. Welsh, D.K., et al., *Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms.* Neuron, 1995. **14**(4): p. 697.
11. Moore, R.Y., J.C. Speh, and R.K. Leak, *Suprachiasmatic nucleus organization.* Cell And Tissue Research, 2002. **309**(1): p. 89-98.
12. Nakamura, W., et al., *Regional pacemakers composed of multiple oscillator neurons in the rat suprachiasmatic nucleus.* European Journal Of Neuroscience, 2001. **14**(4): p. 666-674.
13. Schwartz, W.J., et al., *Differential regulation of fos family genes in the ventrolateral and dorsomedial subdivisions of the rat suprachiasmatic nucleus.* Neuroscience, 2000. **98**(3): p. 535-547.
14. Chambille, I., S. Doyle, and J. Serviere, *Photic Induction And Circadian Expression Of Fos-Like Protein - Immunohistochemical Study In The Retina And Suprachiasmatic Nuclei Of Hamster.* Brain Research, 1993. **612**(1-2): p. 138-150.
15. Guido, M.E., et al., *Daily Rhythm of Spontaneous Immediate-Early Gene Expression in the Rat Suprachiasmatic Nucleus.* Journal of Biological Rhythms, 1999. **14**(4): p. 275-280.
16. Guido, M.E., B. Rusak, and H.A. Robertson, *Spontaneous circadian and light-induced expression of junB mRNA in the hamster suprachiasmatic nucleus.* Brain Research, 1996. **732**(1-2): p. 215-222.

17. Obrietan, K., S. Impey, and D.R. Storm, *Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei.* Nature Neuroscience, 1998. **1**(8): p. 693-700.
18. Gau, D., et al., *Phosphorylation of CREB Ser142 regulates light-induced phase shifts of the circadian clock.* Neuron, 2002. **34**(2): p. 245-252.
19. Obrietan, K., et al., *Circadian regulation of cAMP response element-mediated gene expression in the Suprachiasmatic nuclei.* Journal Of Biological Chemistry, 1999. **274**(25): p. 17748-17756.
20. Coogan, A.N. and H.D. Piggins, *Circadian and photic regulation of phosphorylation of ERK1/2 and Elk-1 in the suprachiasmatic nuclei of the Syrian hamster.* Journal Of Neuroscience, 2003. **23**(7): p. 3085-3093.
21. Hamada, T., et al., *Expression of Period genes: Rhythmic and nonrhythmic compartments of the suprachiasmatic nucleus pacemaker.* Journal Of Neuroscience, 2001. **21**(19): p. 7742-7750.
22. Hastings, M.H. and E.D. Herzog, *Clock Genes, Oscillators, and Cellular Networks in the Suprachiasmatic Nuclei.* Journal of Biological Rhythms, 2004. **19**(5): p. 400-413.
23. Karatsoreos, I.N., et al., *Phenotype Matters: Identification of Light-Responsive Cells in the Mouse Suprachiasmatic Nucleus.* The Journal of Neuroscience, 2004. **24**(1): p. 68-75.
24. Yamaguchi, S., et al., *Synchronization of cellular clocks in the suprachiasmatic nucleus.* Science, 2003. **302**(5649): p. 1408-1412.
25. Schaap, J., et al., *Heterogeneity of rhythmic suprachiasmatic nucleus neurons: Implications, for circadian waveform and photoperiodic encoding.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 2003. **100**(26): p. 15994-15999.
26. Brown, T.M., A.T. Hughes, and H.D. Piggins, *Gastrin-releasing peptide promotes suprachiasmatic nuclei cellular rhythmicity in the absence of vasoactive intestinal polypeptide-VPAC(2) receptor signaling.* Journal Of Neuroscience, 2005. **25**(48): p. 11155-11164.
27. Liu, C. and S.M. Reppert, *GABA synchronizes clock cells within the suprachiasmatic circadian clock.* Neuron, 2000. **25**(1): p. 123-128.
28. Aton, S.J., et al., *GABA and G(i/o) differentially control circadian rhythms and synchrony in clock neurons.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 2006. **103**(50): p. 19188-19193.
29. Colwell, C.S., *Rhythmic coupling among cells in the suprachiasmatic nucleus.* Journal Of Neurobiology, 2000. **43**(4): p. 379-388.
30. Dardente, H., et al., *Daily and circadian expression of neuropeptides in the suprachiasmatic nuclei of nocturnal and diurnal rodents.* Molecular Brain Research, 2004. **124**(2): p. 143-151.
31. Kalamatianos, T., et al., *Expression of VIP and/or PACAP receptor mRNA in peptide synthesizing cells within the suprachiasmatic nucleus of the rat and in its efferent target sites.* The Journal of Comparative Neurology, 2004. **475**(1): p. 19.
32. Harmar, A.J., et al., *The VPAC(2) receptor is essential for circadian function in the mouse suprachiasmatic nuclei.* Cell, 2002. **109**(4): p. 497-508.
33. Reed, H.E., et al., *Vasoactive intestinal polypeptide (VIP) phase-shifts the rat suprachiasmatic nucleus clock in vitro.* European Journal Of Neuroscience, 2001. **13**(4): p. 839-843.

34. Aton, S.J., et al., *Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons.* Nature Neuroscience, 2005. **8**(4): p. 476-483.
35. Maywood, E.S., et al., *Synchronization and maintenance of timekeeping in suprachiasmatic circadian clock cells by neuropeptidergic signaling.* Current Biology, 2006. **16**(6): p. 599-605.
36. Harmar, A.J., et al., *The VPAC2 Receptor Is Essential for Circadian Function in the Mouse Suprachiasmatic Nuclei.* Cell, 2002. **109**(4): p. 497.
37. Colwell, C.S., et al., *Disrupted circadian rhythms in VIP- and PHI-deficient mice.* American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 2003. **285**(5): p. R939-R949.
38. Maywood, E.S., et al., *Synchronization and Maintenance of Timekeeping in Suprachiasmatic Circadian Clock Cells by Neuropeptidergic Signaling.* Current Biology, 2006. **16**(6): p. 599.
39. Cutler, D.J., et al., *The mouse VPAC2 receptor confers suprachiasmatic nuclei cellular rhythmicity and responsiveness to vasoactive intestinal polypeptide in vitro.* European Journal of Neuroscience, 2003. **17**(2): p. 197.
40. Piggins, H.D., M.C. Antle, and B. Rusak, *Neuropeptides phase shift the mammalian circadian pacemaker.* The Journal of Neuroscience, 1995. **15**(8): p. 5612-5622.
41. McArthur, A.J., et al., *Gastrin-Releasing Peptide Phase-Shifts Suprachiasmatic Nuclei Neuronal Rhythms In Vitro.* The Journal of Neuroscience, 2000. **20**(14): p. 5496-5502.
42. Aida, R., et al., *Gastrin-Releasing Peptide Mediates Photic Entrainable Signals to Dorsal Subsets of Suprachiasmatic Nucleus via Induction ofPeriod Gene in Mice.* Molecular Pharmacology, 2002. **61**(1): p. 26-34.
43. Long, M.A., et al., *Electrical synapses coordinate activity in the suprachiasmatic nucleus.* Nat Neurosci, 2005. **8**(1): p. 61.
44. Takahashi, J.S., *Finding new clock components: past and future.* J Biol Rhythms, 2004. **19**(5): p. 339-47.
45. Feldman, J.F. and M.N. Hoyle, *Isolation of circadian clock mutants of Neurospora crassa.* Genetics, 1973. **75**(4): p. 605-13.
46. McClung, C.R., B.A. Fox, and J.C. Dunlap, *The Neurospora clock gene frequency shares a sequence element with the Drosophila clock gene period.* Nature, 1989. **339**(6225): p. 558-62.
47. Konopka, R.J. and S. Benzer, *Clock mutants of Drosophila melanogaster.* Proc Natl Acad Sci U S A, 1971. **68**(9): p. 2112-6.
48. Zehring, W.A., et al., *P-element transformation with period locus DNA restores rhythmicity to mutant, arrhythmic Drosophila melanogaster.* Cell, 1984. **39**(2 Pt 1): p. 369-76.
49. Shearman, L.P., et al., *Interacting molecular loops in the mammalian circadian clock.* Science, 2000. **288**(5468): p. 1013-9.
50. Kondo, T. and M. Ishiura, *The circadian clock of cyanobacteria.* Bioessays, 2000. **22**(1): p. 10-5.
51. Sato TK, Y.R., Ukai H, Baggs JE, Miraglia LJ, Kobayashi TJ, Welsh DK, Kay SA, Ueda HR, Hogenesch JB., *Feedback repression is required for mammalian circadian clock function.* Nat Genet., 2006. **38**(3): p. 312-9.
52. Debruyne, J.P., et al., *A clock shock: mouse CLOCK is not required for circadian oscillator function.* Neuron, 2006. **50**(3): p. 465-77.

53. Sato, T.K., et al., *Feedback repression is required for mammalian circadian clock function.* Nat Genet, 2006. **38**(3): p. 312-9.
54. Zhao WN, M.N., Yang FC, Staknis D, Gekakis N, Maier B, Reischl S, Kramer A, Weitz CJ., *CIPC is a mammalian circadian clock protein without invertebrate homologues.* Nat Cell Biol., 2007. **9**(3): p. 286-275.
55. Preitner, N., et al., *The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator.* Cell, 2002. **110**(2): p. 251-60.
56. Guillaumond, F., et al., *Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors.* J Biol Rhythms, 2005. **20**(5): p. 391-403.
57. Ko, C.H. and J.S. Takahashi, *Molecular components of the mammalian circadian clock.* Human Molecular Genetics, 2006. **15**(suppl 2): p. R271-R277.
58. Gallego, M. and D.M. Virshup, *Post-translational modifications regulate the ticking of the circadian clock.* Nat Rev Mol Cell Biol, 2007. **8**(2): p. 139-48.
59. Godinho, S.I., et al., *The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period.* Science, 2007. **316**(5826): p. 897-900.
60. Siepka, S.M., et al., *Circadian mutant Overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression.* Cell, 2007. **129**(5): p. 1011-23.
61. Ripperger, J.A. and U. Schibler, *Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions.* Nat Genet, 2006. **38**(3): p. 369-74.
62. Doi, M., J. Hirayama, and P. Sassone-Corsi, *Circadian regulator CLOCK is a histone acetyltransferase.* Cell, 2006. **125**(3): p. 497-508.
63. Nakahata, Y., et al., *The NAD(+)-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control.* Cell, 2008. **134**(2): p. 329-340.
64. Asher, G., et al., *SIRT1 regulates circadian clock gene expression through PER2 deacetylation.* Cell, 2008. **134**(2): p. 317-328.
65. Cardone, L., et al., *Circadian clock control by SUMOylation of BMAL1.* Science, 2005. **309**(5739): p. 1390-1394.
66. Provencio, I., et al., *Melanopsin: An opsin in melanophores, brain, and eye.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 1998. **95**(1): p. 340-345.
67. Provencio, I., et al., *A novel human opsin in the inner retina.* Journal Of Neuroscience, 2000. **20**(2): p. 600-605.
68. Gooley, J.J., et al., *Melanopsin in cells of origin of the retinohypothalamic tract.* Nature Neuroscience, 2001. **4**(12): p. 1165-1165.
69. Hattar, S., et al., *Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice.* Nature, 2003. **424**(6944): p. 76-81.
70. Johnson, R.F., R.Y. Moore, and L.P. Morin, *Lateral Geniculate Lesions Alter Circadian Activity Rhythms In The Hamster.* Brain Research Bulletin, 1989. **22**(2): p. 411-422.
71. Card, J.P. and R.Y. Moore, *Ventral Lateral Geniculate-Nucleus Efferents To The Rat Suprachiasmatic Nucleus Exhibit Avian Pancreatic Polypeptide-Like Immunoreactivity.* Journal Of Comparative Neurology, 1982. **206**(4): p. 390-396.
72. Zhang, D.X. and B. Rusak, *Photic Sensitivity Of Geniculate Neurons That Project To The Suprachiasmatic Nuclei Or The Contralateral Geniculate.* Brain Research, 1989. **504**(1): p. 161-164.

73. Harrington, M.E. and B. Rusak, *Lesions of the thalamic intergeniculate leaflet alter hamster circadian rhythms.* Journal of biological rhythms, 1986. **1**(4): p. 309-25.
74. Pickard, G.E., M.R. Ralph, and M. Menaker, *The intergeniculate leaflet partially mediates effects of light on circadian rhythms.* Journal of biological rhythms, 1987. **2**(1): p. 35-56.
75. Moore, R.Y., A.E. Halaris, and B.E. Jones, *Serotonin Neurons Of Midbrain Raphe - Ascending Projections.* Journal Of Comparative Neurology, 1978. **180**(3): p. 417-&.
76. Vandekar, L.D. and S.A. Lorens, *Differential Serotonergic Innervation Of Individual Hypothalamic Nuclei And Other Forebrain Regions By The Dorsal And Median Midbrain Raphe Nuclei.* Brain Research, 1979. **162**(1): p. 45-54.
77. Block, M. and I. Zucker, *Circadian rhythms of rat locomotor activity after lesions of the midbrain raphe nuclei.* Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology, 1976. **109**(3): p. 235.
78. Levine, J.D., et al., *Circadian Activity Rhythms In Rats With Midbrain Raphe Lesions.* Brain Research, 1986. **384**(2): p. 240-249.
79. Borbely, A.A., J.P. Huston, and P.G. Waser, *Physiological And Behavioral Effects Of Parachlorophenylalanine In Rat.* Psychopharmacologia, 1973. **31**(2): p. 131-142.
80. Duncan, W.C., et al., *Chronic Clorgyline Treatment of Syrian Hamsters: An Analysis of Effects on the Circadian Pacemaker.* Journal of Biological Rhythms, 1988. **3**(4): p. 305-322.
81. Challet, E. and P. Pevet, *Interactions between photic and nonphotic stimuli to synchronize the master circadian clock in mammals.* Frontiers In Bioscience, 2003. **8**: p. S246-S257.
82. Ebling, F.J.P., *The role of glutamate in the photic regulation of the suprachiasmatic nucleus.* Progress In Neurobiology, 1996. **50**(2-3): p. 109-&.
83. Vaudry, D., et al., *Pituitary adenylate cyclase-activating polypeptide and its receptors: From structure to functions.* Pharmacological Reviews, 2000. **52**(2): p. 269-324.
84. Harrington, M.E., et al., *Pituitary adenylate cyclase activating peptide phase shifts circadian rhythms in a manner similar to light.* Journal Of Neuroscience, 1999. **19**(15): p. 6637-6642.
85. Chen, D., et al., *PACAP: A pivotal modulator of glutamatergic regulation of the suprachiasmatic circadian clock.* Society for Neuroscience Abstracts, 1999. **25**(1-2): p. 1889.
86. Hannibal, J., et al., *PACAP and glutamate are co-stored in the retinohypothalamic tract.* Journal Of Comparative Neurology, 2000. **418**(2): p. 147-155.
87. Gannon, R.L. and M.A. Rea, *In situ hybridization of antisense mRNA oligonucleotides for AMPA, NMDA and metabotropic glutamate receptor subtypes in the rat suprachiasmatic nucleus at different phases of the circadian cycle.* Brain research. Molecular brain research, 1994. **23**(4): p. 338-44.
88. Rea, M.A., *Photic entrainment of circadian rhythms in rodents.* Chronobiology International, 1998. **15**(5): p. 395-423.
89. Mintz, E.M., et al., *Activation of NMDA Receptors in the Suprachiasmatic Nucleus Produces Light-Like Phase Shifts of the Circadian Clock In Vivo.* The Journal of Neuroscience, 1999. **19**(12): p. 5124-5130.

90. Colwell, C.S. and M. Menaker, *Nmda As Well As Non-Nmda Receptor Antagonists Can Prevent The Phase-Shifting Effects Of Light On The Circadian System Of The Golden-Hamster.* Journal Of Biological Rhythms, 1992. **7**(2): p. 125-136.
91. Marchant, E.G., N.V. Watson, and R.E. Mistlberger, *Both neuropeptide Y and serotonin are necessary for entrainment of circadian rhythms in mice by daily treadmill running schedules.* Journal Of Neuroscience, 1997. **17**(20): p. 7974-7987.
92. Liou, S.Y., et al., *Optic nerve stimulation-induced increase of release of 3h-glutamate and 3h-aspartate but not 3h-gaba from the suprachiasmatic nucleus in slices of rat hypothalamus.* Brain Research Bulletin, 1986. **16**(4): p. 527.
93. Moffett, J.R., et al., *N-Acetylaspartylglutamate - A Transmitter Candidate For The Retinohypothalamic Tract.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 1990. **87**(20): p. 8065-8069.
94. Jacobs, E.H., A. Yamatodani, and H. Timmerman, *Is histamine the final neurotransmitter in the entrainment of circadian rhythms in mammals?* Trends in Pharmacological Sciences, 2000. **21**(8): p. 293.
95. Kim, D.Y., et al., *Substance P plays a critical role in photic resetting of the circadian pacemaker in the rat hypothalamus.* Journal Of Neuroscience, 2001. **21**(11): p. 4026-4031.
96. Goto, K., D.L. Lavalmartin, and L.N. Edmunds, *Biochemical Modeling Of An Autonomously Oscillatory Circadian Clock In Euglena.* Science, 1985. **228**(4705): p. 1284-1288.
97. Bollig, I., et al., *Effects of cAMP, theophylline, imidazole, and 4-(3,4-dimethoxybenzyl)-2-imidazolidone on the leaf movement rhythm of <i>Trifolium repens</i>—a test of the cAMP-hypothesis of circadian rhythms.* Planta, 1978. **141**(2): p. 225.
98. Nakashima, H., *Calcium Inhibits Phase-Shifting Of The Circadian Conidiation Rhythm Of Neurospora-Crassa By The Calcium Ionophore A23187.* Plant Physiology, 1984. **74**(2): p. 268-271.
99. Colwell, C.S., *Circadian modulation of calcium levels in cells in the suprachiasmatic nucleus.* European Journal Of Neuroscience, 2000. **12**(2): p. 571-576.
100. Golombek, D.A., et al., *From light to genes: Moving the hands of the circadian clock.* Frontiers in Bioscience, 2003. **8**(Cited January 9, 2003): p. 285-293.
101. Meijer, J.H., et al., *The effects of glutamate on membrane potential and discharge rate of suprachiasmatic neurons.* Brain Research, 1993. **603**(2): p. 284.
102. Lundkvist, G.B., K. Kristensson, and R.H. Hill, *The Suprachiasmatic nucleus exhibits diurnal variations in spontaneous excitatory postsynaptic activity.* Journal Of Biological Rhythms, 2002. **17**(1): p. 40-51.
103. Michel, S., J. Itri, and C.S. Colwell, *Excitatory mechanisms in the suprachiasmatic nucleus: The role of AMPA/KA glutamate receptors.* Journal Of Neurophysiology, 2002. **88**(2): p. 817-828.
104. Abe, H., B. Rusak, and H.A. Robertson, *Nmda And Non-Nmda Receptor Antagonists Inhibit Photic Induction Of Fos Protein In The Hamster Suprachiasmatic Nucleus.* Brain Research Bulletin, 1992. **28**(5): p. 831-835.
105. Nahm, S.-S., et al., *Circadian Regulation and Function of Voltage-Dependent Calcium Channels in the Suprachiasmatic Nucleus.* The Journal of Neuroscience, 2005. **25**(40): p. 9304-9308.

106. Ginty, D.D., et al., *Regulation Of Creb Phosphorylation In The Suprachiasmatic Nucleus By Light And A Circadian Clock.* Science, 1993. **260**(5105): p. 238-241.
107. O'Neill, J.S., et al., *cAMP-Dependent Signaling as a Core Component of the Mammalian Circadian Pacemaker.* Science, 2008. **320**(5878): p. 949-953.
108. Ferreyra, G.A. and D.A. Golombek, *Cyclic AMP and protein kinase A rhythmicity in the mammalian suprachiasmatic nuclei.* Brain Research, 2000. **858**(1): p. 33-39.
109. Prosser, R.A. and M.U. Gillette, *The Mammalian Circadian Clock In The Suprachiasmatic Nuclei Is Reset Invitro By Camp.* Journal Of Neuroscience, 1989. **9**(3): p. 1073-1081.
110. Tischkau, S.A., et al., *Ca2+/cAMP response element-binding protein (CREB)-dependent activation of Per1 is required for light-induced signaling in the Suprachiasmatic nucleus circadian clock.* Journal Of Biological Chemistry, 2003. **278**(2): p. 718-723.
111. Nomura, K., et al., *Involvement of calcium/calmodulin-dependent protein kinase II in the induction of mPer1.* Journal Of Neuroscience Research, 2003. **72**(3): p. 384-392.
112. Travnickova-Bendova, Z., et al., *Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 2002. **99**(11): p. 7728-7733.
113. Deutsch, P.J., et al., *Structural Determinants For Transcriptional Activation By Camp-Responsive Dna Elements.* Journal Of Biological Chemistry, 1988. **263**(34): p. 18466-18472.
114. Butcher, G.Q., et al., *Light stimulates MSK1 activation in the suprachiasmatic nucleus via a PACAP-ERK/MAP kinase-dependent mechanism.* Journal Of Neuroscience, 2005. **25**(22): p. 5305-5313.
115. Butcher, G.Q., et al., *Light- and clock-dependent regulation of ribosomal S6 kinase activity in the suprachiasmatic nucleus.* European Journal Of Neuroscience, 2004. **19**(4): p. 907-915.
116. Sanada, K., et al., *Role of circadian activation of mitogen-activated protein kinase in chick pineal clock oscillation.* Journal Of Neuroscience, 2000. **20**(3): p. 986-991.
117. Pizzio, G.A., et al., *Circadian and photic regulation of ERK, JNK and p38 in the hamster SCN.* Neuroreport, 2003. **14**(11): p. 1417-1419.
118. Cao, R., et al., *Circadian Regulation Of Mammalian Target Of Rapamycin Signaling In The Mouse Suprachiasmatic Nucleus.* Neuroscience. **181**: p. 79-88.
119. Eckel-Mahan, K.L., et al., *Circadian oscillation of hippocampal MAPK activity and cAMP: implications for memory persistence.* Nature Neuroscience, 2008. **11**(9): p. 1074-1082.
120. Agostino, P.V., et al., *Diurnal, circadian and photic regulation of calcium/calmodulin-dependent kinase II and neuronal nitric oxide synthase in the hamster suprachiasmatic nuclei.* Neurochemistry International, 2004. **44**(8): p. 617.
121. Fukushima, T., et al., *The involvement of calmodulin and Ca2+/calmodulin-dependent protein kinase II in the circadian rhythms controlled by the suprachiasmatic nucleus.* Neuroscience Letters, 1997. **227**(1): p. 45-48.
122. Smolenski, A., et al., *Functional analysis of cGMP-dependent protein kinases I and II as mediators of NO/cGMP effects.* Naunyn-Schmiedebergs Archives Of Pharmacology, 1998. **358**(1): p. 134-139.
123. Ding, J.M., et al., *Resetting The Biological Clock - Mediation Of Nocturnal Circadian Shifts By Glutamate And No.* Science, 1994. **266**(5191): p. 1713-1717.

124. Melo, L., D.A. Golombek, and M.R. Ralph, *Regulation of circadian photic responses by nitric oxide.* Journal Of Biological Rhythms, 1997. **12**(4): p. 319-326.
125. Tischkau, S.A., et al., *Circadian clock-controlled regulation of cGMP-protein kinase G in the nocturnal domain.* Journal Of Neuroscience, 2003. **23**(20): p. 7543-7550.
126. Ferreyra, G.A. and D.A. Golombek, *Rhythmicity of the cGMP-related signal transduction pathway in the mammalian circadian system.* American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 2001. **280**(5): p. R1348-R1355.
127. Weber, E.T., R.L. Gannon, and M.A. Rea, *Cgmp-Dependent Protein-Kinase Inhibitor Blocks Light-Induced Phase Advances Of Circadian-Rhythms In-Vivo.* Neuroscience Letters, 1995. **197**(3): p. 227-230.
128. Ding, J.M., et al., *A neuronal ryanodine receptor mediates light-induced phase delays of the circadian clock.* Nature, 1998. **394**(6691): p. 381-384.
129. Diaz-Munoz, M., et al., *Circadian modulation of the ryanodine receptor type 2 in the SCN of rodents.* Neuroreport, 1999. **10**(3): p. 481-486.
130. Colbran, J.L., Roach, P.J., Fiol, C.J., Dixon, J.E., Andrisani, O.M., Corbin, J.D., *cAMP-dependent protein kinase, but not the cGMP-dependent enzyme, rapidly phosphorylates delta-CREB, and a synthetic delta-CREB peptide.* Biochem. Cell Biol., 1992. **70**: p. 1277- 1282.
131. Mistlberger, R.E., *Effects Of Daily Schedules Of Forced Activity On Free-Running Rhythms In The Rat.* Journal Of Biological Rhythms, 1991. **6**(1): p. 71-80.
132. Edgar, D.M. and W.C. Dement, *Regularly Scheduled Voluntary Exercise Synchronizes The Mouse Circadian Clock.* American Journal Of Physiology, 1991. **261**(4): p. R928-R933.
133. Mrosovsky, N., et al., *Behavioral Entrainment Of Circadian-Rhythms.* Experientia, 1989. **45**(8): p. 696-702.
134. Laemle, L.K. and J.E. Ottenweller, *Nonphotic entrainment of activity and temperature rhythms in anophthalmic mice.* Physiology & Behavior, 1999. **66**(3): p. 461-471.
135. Laemle, L.K., C. Fugaro, and T. Bentley, *The geniculohypothalamic pathway in a congenitally anophthalmic mouse.* Brain Research, 1993. **618**(2): p. 352.
136. Krieger, D.T., *Food And Water Restriction Shifts Corticosterone, Temperature, Activity And Brain Amine Periodicity.* Endocrinology, 1974. **95**(5): p. 1195-1201.
137. Mistlberger, R.E., *Circadian Food-Anticipatory Activity - Formal Models And Physiological-Mechanisms.* Neuroscience And Biobehavioral Reviews, 1994. **18**(2): p. 171-195.
138. Krieger, D.T., H. Hauser, and L.C. Krey, *Suprachiasmatic nuclear lesions do not abolish food-shifted circadian adrenal and temperature rhythmicity.* Science, 1977. **197**(4301): p. 398-399.
139. Phillips, J.L.M. and P.J. Mikulka, *Effects Of Restricted Food Access Upon Locomotor-Activity In Rats With Suprachiasmatic Nucleus Lesions.* Physiology & Behavior, 1979. **23**(2): p. 257-262.
140. Honma, S., K.I. Honma, and T. Hiroshige, *Methamphetamine Induced Locomotor Rhythm Entrains To Restricted Daily Feeding In Scn Lesioned Rats.* Physiology & Behavior, 1989. **45**(5): p. 1057-1065.
141. Stephan, F.K., J.M. Swann, and C.L. Sisk, *Entrainment Of Circadian-Rhythms By Feeding Schedules In Rats With Suprachiasmatic Lesions.* Behavioral And Neural Biology, 1979. **25**(4): p. 545-554.

142. Angeles-Castellanos, M., R. Aguilar-Roblero, and C. Escobar, *c-Fos expression in hypothalamic nuclei of food-entrained rats.* American Journal Of Physiology-Regulatory Integrative And Comparative Physiology, 2004. **286**(1): p. R158-R165.
143. Mendoza, J., M. Angeles-Castellanos, and C. Escobar, *Entrainment by a palatable meal induces food-anticipatory activity and c-Fos expression in reward-related areas of the brain.* Neuroscience, 2005. **133**(1): p. 293-303.
144. Damiola, F., et al., *Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus.* Genes & Development, 2000. **14**(23): p. 2950-2961.
145. Challet, E., et al., *Phase-advanced daily rhythms of melatonin, body temperature, and locomotor activity in food-restricted rats fed during daytime.* Journal Of Biological Rhythms, 1997. **12**(1): p. 65-79.
146. Caldelas, I., et al., *Timed hypocaloric feeding and melatonin synchronize the suprachiasmatic clockwork in rats, but with opposite timing of behavioral output.* European Journal Of Neuroscience, 2005. **22**(4): p. 921-929.
147. Lamont, E.W., et al., *Daily restricted feeding rescues a rhythm of Period2 expression in the arrhythmic suprachiasmatic nucleus.* Neuroscience, 2005. **132**(2): p. 245-248.
148. Guilding, C. and H.D. Piggins, *Challenging the omnipotence of the suprachiasmatic timekeeper: are circadian oscillators present throughout the mammalian brain?* European Journal of Neuroscience, 2007. **25**(11): p. 3195.
149. Balsalobre, A., F. Damiola, and U. Schibler, *A serum shock induces circadian gene expression in mammalian tissue culture cells.* Cell, 1998. **93**(6): p. 929-937.
150. Oishi, K., et al., *Humoral signals mediate the circadian expression of rat period homologue (rPer2) mRNA in peripheral tissues.* Neuroscience Letters, 1998. **256**(2): p. 117-119.
151. Silver, R., et al., *A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms.* Nature, 1996. **382**(6594): p. 810-813.
152. Kalsbeek, A., et al., *Vasopressin-Containing Neurons Of The Suprachiasmatic Nuclei Inhibit Corticosterone Release.* Brain Research, 1992. **580**(1-2): p. 62-67.
153. Kalsbeek, A. and R.M. Buijs, *Peptidergic Transmitters Of The Suprachiasmatic Nuclei And The Control Of Circadian Rhythmicity*, in *Peptidergic Neuron*. 1992. p. 321-333.
154. Tanaka, M., et al., *Direct retinal projections to GRP neurons in the suprachiasmatic nucleus of the rat.* Neuroreport, 1997. **8**(9-10): p. 2187-2191.
155. Kramer, A., et al., *Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling.* Science, 2001. **294**(5551): p. 2511-2515.
156. Reghunandanan, V. and R. Reghunandanan, *Neurotransmitters of the suprachiasmatic nuclei.* Journal of circadian rhythms, 2006. **4**: p. 2.
157. Buijs, R.M., J. Wortel, and Y.-X. Hou, *Colocalization of γ-aminobutyric acid with vasopressin, vasoactive intestinal peptide, and somatostatin in the rat suprachiasmatic nucleus.* The Journal of Comparative Neurology, 1995. **358**(3): p. 343.
158. Hermes, M.L., et al., *GABA and glutamate mediate rapid neurotransmission from suprachiasmatic nucleus to hypothalamic paraventricular nucleus in rat.* The Journal of Physiology, 1996. **496**(Pt 3): p. 749-757.
159. Moore, R.Y., *Organization And Function Of A Central Nervous-System Circadian Oscillator - The Suprachiasmatic Hypothalamic Nucleus.* Federation Proceedings, 1983. **42**(11): p. 2783-2789.

160. Bremner, W.J., M.V. Vitiello, and P.N. Prinz, *Loss Of Circadian Rhythmicity In Blood Testosterone Levels With Aging In Normal Men.* Journal Of Clinical Endocrinology & Metabolism, 1983. **56**(6): p. 1278-1281.
161. Montanini, V., Simoni, M., Chiossi, G., Baraghini, G. F., Velardo, A., Baraldi, E., and Marrama, P., *Age-related changes in plasma dehydroepiandrosterone sulphate, cortisol, testosterone and free testosterone circadian rhythms in adult men.* Hormone Research, 1988. **29**: p. 1-6.
162. Allenrowlands, C.F., et al., *Circadian Rhythmicity Of Acth And Corticosterone In The Rat.* Journal Of Endocrinological Investigation, 1980. **3**(4): p. 371-377.
163. Buijs, R.M., et al., *The biological clock tunes the organs of the body: timing by hormones and the autonomic nervous system.* Journal Of Endocrinology, 2003. **177**(1): p. 17-26.
164. Pevet, P. and E. Challet, *Melatonin: both master clock output and internal time-giver in the circadian clocks network.* Journal of physiology, Paris. **105**(4-6): p. 170-82.
165. Storch, K.F., et al., *Extensive and divergent circadian gene expression in liver and heart.* Nature, 2002. **417**(6884): p. 78-83.
166. Akhtar, R.A., et al., *Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus.* Current Biology, 2002. **12**(7): p. 540-550.
167. Yamamoto, T., et al., *Transcriptional oscillation of canonical clock genes in mouse peripheral tissues.* Bmc Molecular Biology, 2004. **5**.
168. Yoo, S.-H., et al., *PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(15): p. 5339-5346.
169. Yagita, K., et al., *Molecular mechanisms of the biological clock in cultured fibroblasts.* Science, 2001. **292**(5515): p. 278-281.
170. Yamazaki, S., et al., *Effects of aging on central and peripheral mammalian clocks.* Proceedings of the National Academy of Sciences, 2002. **99**(16): p. 10801-10806.
171. Welsh, D.K., et al., *Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression.* Current Biology, 2004. **14**(24): p. 2289-2295.
172. Nagoshi, E., et al., *Circadian gene expression in individual fibroblasts: Cell-autonomous and self-sustained oscillators pass time to daughter cells.* Cell, 2004. **119**(5): p. 693-705.
173. Guo, H.G., et al., *Suprachiasmatic regulation of circadian rhythms of gene expression in hamster peripheral organs: Effects of transplanting the pacemaker.* Journal Of Neuroscience, 2006. **26**(24): p. 6406-6412.
174. Tosini, G. and M. Menaker, *Circadian rhythms in cultured mammalian retina.* Science, 1996. **272**(5260): p. 419-421.
175. Abraham, U., et al., *Independent circadian oscillations of Period1 in specific brain areas in vivo and in vitro.* Journal Of Neuroscience, 2005. **25**(38): p. 8620-8626.
176. Rosenfeld, P., et al., *Ontogeny of corticosteroid receptors in the brain.* Cell Mol Neurobiol, 1993. **13**(4): p. 295-319.
177. Balsalobre, A., L. Marcacci, and U. Schibler, *Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts.* Curr Biol, 2000. **10**(20): p. 1291-4.

178. Le Minh N., D.F., Tronche F., SchutzG., and Schibler U., *Glucocorticoid hormones inhibit food-induced phase shifting of peripheral circadian oscillators.* EMBO J., 2001. **20**(7128).
179. Stokkan KA, Y.S., Tei H, Sakaki Y, Menaker M., *Entrainment of the circadian clock in the liver by feeding.* Science, 2001. **291**(5503): p. 490-3.
180. Hirota T, O.T., Kokame K, Shirotani-Ikejima H, Miyata T, Fukada Y., *Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts.* J Biol Chem., 2002. **277**(46): p. 44244-51.
181. van der Veen DR, M.N., Gos P, Arneric M, Gerkema MP, Schibler U., *Impact of behavior on central and peripheral circadian clocks in the common vole Microtus arvalis, a mammal with ultradian rhythms.* Proc Natl Acad Sci U S A., 2006. **103**(19): p. 3393-8.
182. Zimmerman, W.F., C.S. Pittendrigh, and T. Pavlidis, *Temperature compensation of the circadian oscillation in drosophila pseudoobscura and its entrainment by temperature cycles.* J Insect Physiol, 1968. **14**(5): p. 669-84.
183. Francis, C.D. and M.L. Sargent, *Effects of Temperature Perturbations on Circadian Conidiation in Neurospora.* Plant Physiol, 1979. **64**(6): p. 1000-1004.
184. Brown, S.A., et al., *Rhythms of mammalian body temperature can sustain peripheral circadian clocks.* Curr Biol, 2002. **12**(18): p. 1574-83.
185. Terazono, H., et al., *Adrenergic regulation of clock gene expression in mouse liver.* Proc Natl Acad Sci U S A, 2003. **100**(11): p. 6795-800.
186. Cuninkova, L. and S.A. Brown, *Peripheral circadian oscillators - Interesting mechanisms and powerful tools.* Molecular And Biophysical Mechanisms Of Arousal, Alertness, And Attention, 2008. **1129**: p. 358-370.
187. Bell-Pedersen, D., et al., *Circadian rhythms from multiple oscillators: lessons from diverse organisms.* Nat Rev Genet, 2005. **6**(7): p. 544.
188. Ralph, M.R. and M. Menaker, *A Mutation Of The Circadian System In Golden-Hamsters.* Science, 1988. **241**(4870): p. 1225-1227.
189. Vitaterna, M.H., et al., *Mutagenesis And Mapping Of A Mouse Gene Clock, Essential For Circadian Behavior.* Science, 1994. **264**(5159): p. 719-725.
190. Jewett, M.E., R.E. Kronauer, and C.A. Czeisler, *Light-induced suppression of endogenous circadian amplitude in humans.* Nature, 1991. **350**(6313): p. 59.
191. Pulivarthy, S.R., et al., *Reciprocity between phase shifts and amplitude changes in the mammalian circadian clock.* Proceedings of the National Academy of Sciences, 2007. **104**(51): p. 20356-20361.
192. Spoelstra, K., et al., *Phase Responses to Light Pulses in Mice Lacking Functional per or cry Genes.* Journal of Biological Rhythms, 2004. **19**(6): p. 518-529.
193. Challet, E., et al., *Light exposure during daytime modulates expression of Per1 and Per2 clock genes in the suprachiasmatic nuclei of mice.* Journal of Neuroscience Research, 2003. **72**(5): p. 629.
194. Klein, D.C., D. Sugden, and J.L. Weller, *Postsynaptic alpha-adrenergic receptors potentiate the beta-adrenergic stimulation of pineal serotonin N-acetyltransferase.* Proceedings of the National Academy of Sciences, 1983. **80**(2): p. 599-603.
195. Vanecek J, S.D., Weller J, Klein DC, *Atypical synergistic b1- and a1-adrenergic regulation of adenosine 39,59-monophosphate in cultured rat pinealocytes.* Endocrinology, 1985. **116**(2167-2173).

196. Roseboom, P.H. and D.C. Klein, *Norepinephrine stimulation of pineal cyclic AMP response element-binding protein phosphorylation: primary role of a beta-adrenergic receptor/cyclic AMP mechanism.* Molecular Pharmacology, 1995. **47**(3): p. 439-449.
197. Tamotsu, S., et al., *Norepinephrine-Induced Phosphorylation Of The Transcription Factor Creb In Isolated Rat Pinealocytes - An Immunocytochemical Study.* Cell And Tissue Research, 1995. **282**(2): p. 219-226.
198. Baler, R., S. Covington, and D.C. Klein, *The Rat Arylalkylamine N-Acetyltransferase Gene Promoter. cAMP ACTIVATION VIA A cAMP-RESPONSIVE ELEMENT-CCAAT COMPLEX.* J. Biol. Chem., 1997. **272**(11): p. 6979-6985.
199. Molina, C.A., et al., *Inducibility and negative autoregulation of CREM: An alternative promoter directs the expression of ICER, an early response repressor.* Cell, 1993. **75**(5): p. 875.
200. Stehle, J.H., et al., *Adrenergic Signals Direct Rhythmic Expression Of Transcriptional Repressor Crem In The Pineal-Gland.* Nature, 1993. **365**(6444): p. 314-320.
201. Maronde, E., et al., *Transcription Factors in Neuroendocrine Regulation: Rhythmic Changes in pCREB and ICER Levels Frame Melatonin Synthesis.* The Journal of Neuroscience, 1999. **19**(9): p. 3326-3336.
202. Reppert, S.M., *Melatonin Receptors: Molecular Biology of a New Family of G Protein-Coupled Receptors.* Journal of Biological Rhythms, 1997. **12**(6): p. 528-531.
203. Pickering, D.S. and L.P. Niles, *Pharmacological Characterization Of Melatonin Binding-Sites In Syrian-Hamster Hypothalamus.* European Journal Of Pharmacology, 1990. **175**(1): p. 71-77.
204. Boutin, J.A., et al., *Molecular tools to study melatonin pathways and actions.* Trends In Pharmacological Sciences, 2005. **26**(8): p. 412-419.
205. Ackermann, K. and J.H. Stehle, *Melatonin synthesis in the human pineal gland: Advantages, implications, and difficulties.* Chronobiology International, 2006. **23**(1-2): p. 369-379.
206. Witt-Enderby, P.A., M.I. Masana, and M.L. Dubocovich, *Physiological exposure to melatonin supersensitizes the cyclic adenosine 3 ',5 '-monophosphate-dependent signal transduction cascade in Chinese hamster ovary cells expressing the human mt(1) melatonin receptor.* Endocrinology, 1998. **139**(7): p. 3064-3071.
207. Masana, M.I. and M.L. Dubocovich, *Melatonin Receptor Signaling: Finding the Path Through the Dark.* Sci. STKE, 2001. **2001**(107): p. pe39-.
208. Witt-Enderby, P.A., et al., *Melatonin induction of filamentous structures in non-neuronal cells that is dependent on expression of the human mt1 melatonin receptor.* Cell Motility And The Cytoskeleton, 2000. **46**(1): p. 28-42.
209. Roka, F., et al., *Tight association of the human Mel(1a)-melatonin receptor and G(i): Precoupling and constitutive activity.* Molecular Pharmacology, 1999. **56**(5): p. 1014-1024.
210. Brydon, L., et al., *Dual signaling of human Mel1a melatonin receptors via G(i2), G(i3), and G(q/11) proteins.* Molecular Endocrinology, 1999. **13**(12): p. 2025-2038.
211. Godson, C. and S.M. Reppert, *The Mel(1a) melatonin receptor is coupled to parallel signal transduction pathways.* Endocrinology, 1997. **138**(1): p. 397-404.
212. Nelson, C.S., J.L. Marino, and C.N. Allen, *Melatonin receptors activate heteromeric G-protein coupled Kir3 channels.* Neuroreport, 1996. **7**(3): p. 717-720.

213. Chan, A.S.L., et al., *Melatonin mt1 and MT2 receptors stimulate c-Jun N-terminal kinase via pertussis toxin-sensitive and -insensitive G proteins.* Cellular Signalling, 2002. **14**(3): p. 249-257.
214. Petit, L., et al., *Differential signaling of human Mel1a and Mel1b melatonin receptors through the cyclic guanosine 3 '-5 '-monophosphate pathway.* Biochemical Pharmacology, 1999. **58**(4): p. 633-639.
215. Wan, Q., et al., *Differential modulation of GABA(A) receptor function by Mel(1a) and Mel(1b) receptors.* Nature Neuroscience, 1999. **2**(5): p. 401-403.
216. Hunt, A.E., et al., *Activation of MT(2) melatonin receptors in rat suprachiasmatic nucleus phase advances the circadian clock.* American Journal Of Physiology-Cell Physiology, 2001. **280**(1): p. C110-C118.
217. Scher, J., et al., *MT(1) melatonin receptor in the human retina: Expression and localization.* Investigative Ophthalmology & Visual Science, 2002. **43**(3): p. 889-897.
218. Meyer, P., et al., *Melatonin MT-1-receptor immunoreactivity in the human eye.* British Journal Of Ophthalmology, 2002. **86**(9): p. 1053-1057.
219. Savaskan, E., et al., *Distribution of melatonin MT1 receptor immunoreactivity in human retina.* Journal Of Histochemistry & Cytochemistry, 2002. **50**(4): p. 519-525.
220. Osborne, N.N. and G. Chidlow, *The Presence Of Functional Melatonin Receptors In The Iris-Ciliary Processes Of The Rabbit Eye.* Experimental Eye Research, 1994. **59**(1): p. 3-9.
221. Shibata, S., V.M. Cassone, and R.Y. Moore, *Effects Of Melatonin On Neuronal-Activity In The Rat Suprachiasmatic Nucleus Invitro.* Neuroscience Letters, 1989. **97**(1-2): p. 140-144.
222. McArthur, A.J., A.E. Hunt, and M.U. Gillette, *Melatonin action and signal transduction in the rat suprachiasmatic circadian clock: Activation of protein kinase C at dusk and dawn.* Endocrinology, 1997. **138**(2): p. 627-634.
223. von Gall, C., et al., *Melatonin limits transcriptional impact of phosphoCREB in the mouse SCN via the Mel1a receptor.* NeuroReport, 2000. **11**(9): p. 1803-1807.
224. Tamarkin, L., C.J. Baird, and O.F. Almeida, *Melatonin: a coordinating signal for mammalian reproduction?* Science, 1985. **227**(4688): p. 714-720.
225. Malpaux, B., et al., *Biology of mammalian photoperiodism and the critical role of the pineal gland and melatonin.* Journal Of Biological Rhythms, 2001. **16**(4): p. 336-347.
226. Soares, J.M., et al., *Functional melatonin receptors in rat ovaries at various stages of the estrous cycle.* Journal Of Pharmacology And Experimental Therapeutics, 2003. **306**(2): p. 694-702.
227. Barrenetxe, J., P. Delagrange, and J. Martínez, *Physiological and metabolic functions of melatonin.* Journal of Physiology and Biochemistry, 2004. **60**(1): p. 61.
228. Peschke, E., et al., *Evidence for a melatonin receptor within pancreatic islets of neonate rats: functional, autoradiographic, and molecular investigations.* Journal Of Pineal Research, 2000. **28**(3): p. 156-164.
229. Zalatan, F., J.A. Krause, and D.E. Blask, *Inhibition of isoproterenol-induced lipolysis in rat inguinal adipocytes in vitro by physiological melatonin via a receptor-mediated mechanism.* Endocrinology, 2001. **142**(9): p. 3783-3790.
230. Masana, M.I., et al., *MT(2) melatonin receptors are present and functional in rat caudal artery.* Journal Of Pharmacology And Experimental Therapeutics, 2002. **302**(3): p. 1295-1302.

231. Maestroni, G.J.M., *MLT and the immune-hematopoietic system*, in *Melatonin After Four Decades: An Assessment Of Its Potential*. 1999. p. 395-405.
232. Pawlikowski, M., K. Winczyk, and M. Karasek, *Oncostatic action of melatonin: facts and question marks.* Neuroendocrinology Letters, 2002. **23**: p. 24-29.
233. Sanchez-Barcelo, E.J., et al., *Melatonin and mammary cancer: a short review.* Endocrine-Related Cancer, 2003. **10**(2): p. 153-159.
234. Lang, U., et al., *Radioimmunological Determination Of Urinary Melatonin In Humans - Correlation With Plasma-Levels And Typical 24-Hour Rhythmicity.* Journal Of Clinical Endocrinology & Metabolism, 1981. **53**(3): p. 645-650.
235. KrÃ¤uchi, K., et al., *Alteration of Internal Circadian Phase Relationships after Morning versus Evening Carbohydrate-Rich Meals in Humans.* Journal of Biological Rhythms, 2002. **17**(4): p. 364-376.
236. Lewy, A.J., et al., *Light Suppresses Melatonin Secretion In Humans.* Science, 1980. **210**(4475): p. 1267-1269.
237. Deacon, S. and J. Arendt, *Posture Influences Melatonin Concentrations In Plasma And Saliva In Humans.* Neuroscience Letters, 1994. **167**(1-2): p. 191-194.
238. Monteleone, P., et al., *Physical Exercise At Night Blunts The Nocturnal Increase Of Plasma Melatonin Levels In Healthy Humans.* Life Sciences, 1990. **47**(22): p. 1989-1995.
239. Shilo, L., et al., *The effects of coffee consumption on sleep and melatonin secretion.* Sleep medicine, 2002. **3**(3): p. 271-3.
240. Hajak, G., et al., *Nocturnal plasma melatonin levels after flunitrazepam administration in healthy subjects.* European Neuropsychopharmacology, 1996. **6**(2): p. 149-153.
241. Yamada, Y., et al., *Prediction of sleep disorders induced by beta-adrenergic receptor blocking agents based on receptor occupancy.* Journal Of Pharmacokinetics And Biopharmaceutics, 1995. **23**(2): p. 131-145.
242. Aschoff, J., *The Circadian Rhythm Of Body Temperature As A Function Of Body Size*, in *Taylor, C. R., K. Johansen And L. Bolis (Ed.). A Companion To Animal Physiology; 5th International Conference On Comparative Physiology, Sandbjerg, Denmark, July 22-26, 1980. Xv+365p. Cambridge University Press: Cambridge, England; New York, N.Y., Usa. Illus.* 1982. p. P173-188.
243. Minors, D.S. and J.M. Waterhouse, *The Use Of Constant Routines In Unmasking The Endogenous Component Of Human Circadian Rhythms.* Chronobiology International, 1984. **1**(3): p. 205-216.
244. Czeisler, C.A., et al., *Bright Light Resets The Human Circadian Pacemaker Independent Of The Timing Of The Sleep-Wake Cycle.* Science, 1986. **233**(4764): p. 667-671.
245. Krauchi, K., C. Cajochen, and A. WirzJustice, *A relationship between heat loss and sleepiness: Effects of postural change and melatonin administration.* Journal Of Applied Physiology, 1997. **83**(1): p. 134-139.
246. Wever, R.A., *Internal Interactions Within The Human Circadian System - The Masking Effect.* Experientia, 1985. **41**(3): p. 332-342.
247. Minors, D.S. and J.M. Waterhouse, *Masking In Humans - The Problem And Some Attempts To Solve It.* Chronobiology International, 1989. **6**(1): p. 29-53.
248. Gander, P.H., L.J. Connell, and R.C. Graeber, *Masking Of The Circadian Rhythms Of Heart Rate And Core Temperature By The Rest-Activity Cycle In Man.* Journal of Biological Rhythms, 1986. **1**(2): p. 119-136.

249. Dijk, D.-J., C. Cajochen, and A.A. BorbÃ©ly, *Effect of a single 3-hour exposure to bright light on core body temperature and sleep in humans.* Neuroscience Letters, 1991. **121**(1â€“2): p. 59.
250. Brown, E.N. and C.A. Czeisler, *The Statistical Analysis of Circadian Phase and Amplitude in Constant-Routine Core-Temperature Data.* Journal of Biological Rhythms, 1992. **7**(3): p. 177-202.
251. Buijs, R.M., et al., *Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway.* European Journal Of Neuroscience, 1999. **11**(5): p. 1535-1544.
252. Linkowski, P., et al., *Twin Study Of The 24-H Cortisol Profile - Evidence For Genetic-Control Of The Human Circadian Clock.* American Journal Of Physiology, 1993. **264**(2): p. E173-E181.
253. Umeda, T., et al., *Use Of Saliva For Monitoring Un-Bound Free Cortisol-Levels In Serum.* Clinica Chimica Acta, 1981. **110**(2-3): p. 245-253.
254. VanCauter, E., R. Leproult, and D.J. Kupfer, *Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol.* Journal Of Clinical Endocrinology & Metabolism, 1996. **81**(7): p. 2468-2473.
255. Weibel, L. and G. Brandenberger, *The start of the quiescent period of cortisol remains phase locked to the melatonin onset despite circadian phase alterations in humans working the night schedule.* Neuroscience Letters, 2002. **318**(2): p. 89-92.
256. Scheer, F. and R.M. Buijs, *Light affects morning salivary cortisol in humans.* Journal Of Clinical Endocrinology & Metabolism, 1999. **84**(9): p. 3395-3398.
257. Leproult, R., et al., *Transition from dim to bright light in the morning induces an immediate elevation of cortisol levels.* Journal Of Clinical Endocrinology & Metabolism, 2001. **86**(1): p. 151-157.
258. Caufriez, A., et al., *Immediate effects of an 8-h advance shift of the rest-activity cycle on 24-h profiles of cortisol.* American Journal Of Physiology-Endocrinology And Metabolism, 2002. **282**(5): p. E1147-E1153.
259. Gronfier, C., et al., *Pulsatile cortisol secretion and EEG delta waves are controlled by two independent but synchronized generators.* American Journal Of Physiology-Endocrinology And Metabolism, 1998. **275**(1): p. E94-E100.
260. Antunes, L.C., et al., *Obesity and shift work: chronobiological aspects.* Nutrition Research Reviews. **23**(1): p. 155-168.
261. Fonken, L.K., et al., *Light at night increases body mass by shifting the time of food intake.* Proceedings Of The National Academy Of Sciences Of The United States Of America. **107**(43): p. 18664-18669.
262. Reddy, A.B., et al., *Circadian Orchestration of the Hepatic Proteome.* Current Biology, 2006. **16**(11): p. 1107.
263. Panda, S., et al., *Coordinated Transcription of Key Pathways in the Mouse by the Circadian Clock.* Cell, 2002. **109**(3): p. 307.
264. Stokkan, K.-A., et al., *Entrainment of the Circadian Clock in the Liver by Feeding.* Science, 2001. **291**(5503): p. 490-493.
265. Dallmann, R., et al., *The human circadian metabolome.* Proceedings of the National Academy of Sciences.
266. Ramsey, K.M., et al., *Circadian Clock Feedback Cycle Through NAMPT-Mediated NAD+ Biosynthesis.* Science, 2009. **324**(5927): p. 651-654.

267. Nakahata, Y., et al., *Circadian Control of the NAD+ Salvage Pathway by CLOCK-SIRT1.* Science, 2009. **324**(5927): p. 654-657.
268. Minami, Y., et al., *Measurement of internal body time by blood metabolomics.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 2009. **106**(24): p. 9890-9895.
269. DeCoursey, P.J., *Daily light sensitivity in a rodent.* Science, 1960. **131**: p. 33-35.
270. Aschoff, J., *Rersponse curves in circadian periodicity.* In J. Aschoff (Ed.), Circadian CLocks, North-Holland, Amsterdam, 1965: p. 95-111.
271. Czeisler, C.A., et al., *Stability, precision, and near-24-hour period of the human circadian pacemaker.* Science, 1999. **284**(5423): p. 2177-81.
272. Carskadon, M.A., et al., *Intrinsic circadian period of adolescent humans measured in conditions of forced desynchrony.* Neurosci Lett, 1999. **260**(2): p. 129-32.
273. Gronfier C, W.K.J., Kronauer RE, Jewett ME, Czeisler CA., *Efficacy of a single sequence of intermittent bright light pulses for delaying circadian phase in humans.* Am J Physiol Endocrinol Metab., 2004. **287**(1): p. E174-81.
274. Duffy, J.F., et al., *Relationship of endogenous circadian melatonin and temperature rhythms to self-reported preference for morning or evening activity in young and older people.* J Investig Med, 1999. **47**(3): p. 141-50.
275. Duffy, J.F., D.W. Rimmer, and C.A. Czeisler, *Association of intrinsic circadian period with morningness-eveningness, usual wake time, and circadian phase.* Behav Neurosci, 2001. **115**(4): p. 895-9.
276. Steven A. Brown, D.K., Amelie Dumas, Pal O. Westermark. Katja ZVanselow, Amely Tilmann-Wahnschaffe, Janspeter Herzel, and Achim Kramer, *Molecular insights into human daily behavior.* submitted, 2007.
277. Khalsa, S.B.S., et al., *A phase response curve to single bright light pulses in human subjects.* Journal Of Physiology-London, 2003. **549**(3): p. 945-952.
278. Saunders, D.S. and E.J. Thomson, *Strong Phase Response Curve For Circadian-Rhythm Of Locomotor-Activity In A Cockroach (Nauphoeta-Cinerea).* Nature, 1977. **270**(5634): p. 241-243.
279. Lewy, A.J., et al., *Melatonin shifts human circadian rhythms according to a phase-response curve.* Chronobiol Int, 1992. **9**(5): p. 380-92.
280. Czeisler, C.A., et al., *Stability, precision, and near-24-hour period of the human circadian pacemaker.* Science, 1999. **284**(5423): p. 2177-2181.
281. Dijk, D.J. and C.A. Czeisler, *Contribution Of The Circadian Pacemaker And The Sleep Homeostat To Sleep Propensity, Sleep Structure, Electroencephalographic Slow Waves, And Sleep Spindle Activity In Humans.* Journal Of Neuroscience, 1995. **15**(5): p. 3526-3538.
282. Hiddinga, A.E., D.G.M. Beersma, and R.H. VandenHoofdakker, *Endogenous and exogenous components in the circadian variation of core body temperature in humans.* Journal Of Sleep Research, 1997. **6**(3): p. 156-163.
283. Czeisler, C.A., et al., *Bright Light Induction Of Strong (Type-0) Resetting Of The Human Circadian Pacemaker.* Science, 1989. **244**(4910): p. 1328-1333.
284. Duffy, J.F. and D.-J. Dijk, *Getting Through to Circadian Oscillators: Why Use Constant Routines?* Journal of Biological Rhythms, 2002. **17**(1): p. 4-13.
285. Wirz-Justice, A., C. Cajochen, and P. Nussbaum, *A schizophrenic patient with an arrhythmic circadian rest-activity cycle.* Psychiatry Research, 1997. **73**(1â€“2): p. 83.

286. Cajochen, C., et al., *Dynamics of frontal EEG activity, sleepiness and body temperature under high and low sleep pressure.* Neuroreport, 2001. **12**(10): p. 2277-2281.
287. Brown, S.A., et al., *The period length of fibroblast circadian gene expression varies widely among human individuals.* Plos Biology, 2005. **3**(10): p. 1813-1818.
288. Brown, S.A., et al., *Molecular insights into human daily behavior.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 2008. **105**(5): p. 1602-1607.
289. Pagani, L., et al., *The Physiological Period Length of the Human Circadian Clock In Vivo Is Directly Proportional to Period in Human Fibroblasts.* Plos One. **5**(10).
290. Liu, C., et al., *Cellular construction of a circadian clock: period determination in the suprachiasmatic nuclei.* Cell, 1997. **91**(6): p. 855-60.
291. Pando, M.P., et al., *Phenotypic rescue of a peripheral clock genetic defect via SCN hierarchical dominance.* Cell, 2002. **110**(1): p. 107-17.
292. Yagita, K., et al., *Molecular mechanisms of the biological clock in cultured fibroblasts.* Science, 2001. **292**(5515): p. 278-81.
293. Yoo, S.H., et al., *PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues.* Proc Natl Acad Sci U S A, 2004. **101**(15): p. 5339-46.
294. Brown, S.A., et al., *The period length of fibroblast circadian gene expression varies widely among human individuals.* PLoS Biol, 2005. **3**(10): p. e338.
295. Liu, A.C., et al., *Intercellular coupling confers robustness against mutations in the SCN circadian clock network.* Cell, 2007. **129**(3): p. 605-16.
296. Maywood, E.S., et al., *Synchronization and maintenance of timekeeping in suprachiasmatic circadian clock cells by neuropeptidergic signaling.* Curr Biol, 2006. **16**(6): p. 599-605.
297. Dey, J., et al., *The tau mutation in the Syrian hamster differentially reprograms the circadian clock in the SCN and peripheral tissues.* J Biol Rhythms, 2005. **20**(2): p. 99-110.
298. Czeisler CA, D.D., Kronauer RE, Brown EN, Duffy JF, Allan JS, Shanahan TL, Rimmer DW, Ronda JM, Mitchell JF, Silva EJ, Emens JS., *Is there an intrinsic period of the circadian clock? Response.* Science, 2000. **288**(5469): p. 1174-5.
299. S., C., *Is there an intrinsic period of the circadian clock?* Science, 2000. **288**(5469): p. 1174-5.
300. Vitaterna, M.H., et al., *The mouse Clock mutation reduces circadian pacemaker amplitude and enhances efficacy of resetting stimuli and phase-response curve amplitude.* Proc Natl Acad Sci U S A, 2006. **103**(24): p. 9327-32.
301. Vanselow, K., et al., *Differential effects of PER2 phosphorylation: molecular basis for the human familial advanced sleep phase syndrome (FASPS).* Genes Dev, 2006. **20**(19): p. 2660-72.
302. Lee, C.C., *Tumor suppression by the mammalian Period genes.* Cancer Causes & Control, 2006. **17**(4): p. 525-530.
303. Stevens, R.G., *Circadian Disruption and Breast Cancer: From Melatonin to Clock Genes.* Epidemiology, 2005. **16**(2): p. 254.
304. Lemmer, B.r., *Chronopharmacology and controlled drug release.* Expert Opinion on Drug Delivery, 2005. **2**(4): p. 667.

305. Mecacci, L. and A. Zani, *Morningness-eveningness preferences and sleep-waking diary data of morning and evening types in student and worker samples.* Ergonomics, 1983. **26**(12): p. 1147.
306. Deurveilher, S. and K. Semba, *Indirect projections from the suprachiasmatic nucleus to major arousal-promoting cell groups in rat: Implications for the circadian control of behavioural state.* Neuroscience, 2005. **130**(1): p. 165.
307. Borbely, A.A., *A two process model of sleep regulation.* Human neurobiology, 1982. **1**(3): p. 195-204.
308. Roenneberg, T., A. Wirz-Justice, and M. Merrow, *Life between clocks: Daily temporal patterns of human chronotypes.* Journal Of Biological Rhythms, 2003. **18**(1): p. 80-90.
309. Franken, P. and D.J. Dijk, *Circadian clock genes and sleep homeostasis.* European Journal of Neuroscience, 2009. **29**(9): p. 1820.
310. Bunger, M.K., et al., *Mop3 is an essential component of the master circadian pacemaker in mammals.* Cell, 2000. **103**(7): p. 1009-1017.
311. Laposky, A., et al., *Deletion of the mammalian circadian clock gene BMAL1/Mop3 alters baseline sleep architecture and the response to sleep deprivation.* Sleep, 2005. **28**(4): p. 395-409.
312. Naylor, E., et al., *The Circadian Clock Mutation Alters Sleep Homeostasis in the Mouse.* The Journal of Neuroscience, 2000. **20**(21): p. 8138-8143.
313. Dudley, C.A., et al., *Altered patterns of sleep and behavioral adaptability in NPAS2-defficient mice.* Science, 2003. **301**(5631): p. 379-383.
314. Franken, P., et al., *NPAS2 as a transcriptional regulator of non-rapid eye movement sleep: Genotype and sex interactions.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 2006. **103**(18): p. 7118-7123.
315. Wisorl, J.P., et al., *A role for cryptochromes in sleep regulation.* Bmc Neuroscience, 2002. **3**.
316. Kopp, C., et al., *Homeostatic sleep regulation is preserved in mPer1 and mPer2 mutant mice.* European Journal Of Neuroscience, 2002. **16**(6): p. 1099-1106.
317. Wisor, J.P., et al., *Sleep deprivation effects on circadian clock gene expression in the cerebral cortex parallel electroencephalographic differences among mouse strains.* Journal Of Neuroscience, 2008. **28**(28): p. 7193-7201.
318. Shiromani, P.J., et al., *Sleep rhythmicity and homeostasis in mice with targeted disruption of mPeriod genes.* American Journal Of Physiology-Regulatory Integrative And Comparative Physiology, 2004. **287**(1): p. R47-R57.
319. Franken, P., et al., *The transcription factor DBP affects circadian sleep consolidation and rhythmic EEG activity.* Journal Of Neuroscience, 2000. **20**(2): p. 617-625.
320. He, Y., et al., *The Transcriptional Repressor DEC2 Regulates Sleep Length in Mammals.* Science, 2009. **325**(5942): p. 866-870.
321. Xu, Y., et al., *Functional consequences of a CKIdelta mutation causing familial advanced sleep phase syndrome.* Nature, 2005. **434**(7033): p. 640-4.
322. Carpen, J.D., et al., *A silent polymorphism in the PER1 gene associates with extreme diurnal preference in humans.* J Hum Genet, 2006. **51**(12): p. 1122-5.
323. Benedetti, F., et al., *Long-term response to lithium salts in bipolar illness is influenced by the glycogen synthase kinase 3-beta -50 T/C SNP.* Neurosci Lett, 2005. **376**(1): p. 51-5.
324. Mansour, H.A., et al., *Association study of eight circadian genes with bipolar I disorder, schizoaffective disorder and schizophrenia.* Genes Brain Behav, 2006. **5**(2): p. 150-7.

325. Ebisawa, T., et al., *Association of structural polymorphisms in the human period3 gene with delayed sleep phase syndrome.* EMBO Rep, 2001. **2**(4): p. 342-6.
326. Johansson, C., et al., *Circadian clock-related polymorphisms in seasonal affective disorder and their relevance to diurnal preference.* Neuropsychopharmacology, 2003. **28**(4): p. 734-9.
327. Zhu, Y., et al., *Ala394Thr polymorphism in the clock gene NPAS2: a circadian modifier for the risk of non-Hodgkin's lymphoma.* Int J Cancer, 2007. **120**(2): p. 432-5.
328. Takao, T., et al., *CLOCK gene T3111C polymorphism is associated with Japanese schizophrenics: a preliminary study.* Eur Neuropsychopharmacol, 2007. **17**(4): p. 273-6.
329. Partonen, T., et al., *Three circadian clock genes Per2, Arntl, and Npas2 contribute to winter depression.* Ann Med, 2007. **39**(3): p. 229-38.
330. Jones, C.R., et al., *Familial advanced sleep-phase syndrome: A short-period circadian rhythm variant in humans.* Nat Med, 1999. **5**(9): p. 1062-5.
331. Toh, K.L., et al., *An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome.* Science, 2001. **291**(5506): p. 1040-3.
332. Lack, L. and H. Wright, *The Effect Of Evening Bright Light In Delaying The Circadian-Rhythms And Lengthening The Sleep Of Early-Morning Awakening Insomniacs.* Sleep, 1993. **16**(5): p. 436-443.
333. Lack, L., et al., *The treatment of early-morning awakening insomnia with 2 evenings of bright light.* Sleep, 2005. **28**(5): p. 616-623.
334. Lack, L.C. and H.R. Wright, *Treating chronobiological components of chronic insomnia.* Sleep Medicine, 2007. **8**(6): p. 637-644.
335. Hughes, R.J., R.L. Sack, and A.J. Lewy, *The role of melatonin and circadian phase in age-related sleep-maintenance insomnia: Assessment in a clinical trial of melatonin replacement.* Sleep, 1998. **21**(1): p. 52-68.
336. Archer, S.N., et al., *A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference.* Sleep, 2003. **26**(4): p. 413-415.
337. Pereira, D.S., et al., *Association of the length polymorphism delayed sleep-phase syndrome: Does in the human Per3 gene with the latitude have an influence upon it?* Sleep, 2005. **28**(1): p. 29-32.
338. Ebisawa, T., et al., *Association of structural polymorphisms in the human period3 gene with delayed sleep phase syndrome.* Embo Reports, 2001. **2**(4): p. 342-346.
339. Katzenberg, D., et al., *A CLOCK polymorphism associated with human diurnal preference.* Sleep, 1998. **21**(6): p. 569-576.
340. Robilliard, D.L., et al., *The 3111 Clock gene polymorphism is not associated with sleep and circadian rhythmicity in phenotypically characterized human subjects.* Journal of Sleep Research, 2002. **11**(4): p. 305.
341. Duffy, J.F., D.W. Rimmer, and C.A. Czeisler, *Association of intrinsic circadian period with morningness-eveningness, usual wake time, and circadian phase.* Behavioral Neuroscience, 2001. **115**(4): p. 895-899.
342. Shimomura, K., et al., *Genome-wide epistatic interaction analysis reveals complex genetic determinants of circadian behavior in mice.* Genome Research, 2001. **11**(6): p. 959-980.
343. Rosenthal, N.E., et al., *Phase-Shifting Effects Of Bright Morning Light As Treatment For Delayed Sleep Phase Syndrome.* Sleep, 1990. **13**(4): p. 354-361.

344. Cole, R.J., et al., *Bright-light mask treatment of delayed sleep phase syndrome.* Journal Of Biological Rhythms, 2002. **17**(1): p. 89-101.
345. Dagan, Y., et al., *Evaluating the role of melatonin in the long-term treatment of delayed sleep phase syndrome (DSPS).* Chronobiology International, 1998. **15**(2): p. 181-190.
346. Miles, L.E., D.M. Raynal, and M.A. Wilson, *Blind man living in normal society has circadian rhythms of 24.9 hours.* Science, 1977. **198**(4315): p. 421-423.
347. Klein T, M.H., Dijk DJ, Kronauer RE, Seely EW, Czeisler CA., *Circadian sleep regulation in the absence of light perception: chronic non-24-hour circadian rhythm sleep disorder in a blind man with a regular 24-hour sleep-wake schedule.* Sleep, 1993. **16**(4): p. 333-43.
348. McArthur, A.J., A.J. Lewy, and R.L. Sack, *Non-24-hour sleep-wake syndrome in a sighted man: Circadian rhythm studies and efficacy of melatonin treatment.* Sleep, 1996. **19**(7): p. 544-553.
349. Lockley, S.W., et al., *Melatonin administration can entrain the free-running circadian system of blind subjects.* Journal Of Endocrinology, 2000. **164**(1): p. R1-R6.
350. Sack, R.L., et al., *Entrainment of free-running circadian rhythms by melatonin in blind people.* New England Journal Of Medicine, 2000. **343**(15): p. 1070-1077.
351. Lewy, A.J., et al., *Pretreatment circadian period in free-running blind people may predict the phase angle of entrainment to melatonin.* Neuroscience Letters, 2001. **313**(3): p. 158-160.
352. Witting, W., et al., *Alterations In The Circadian Rest-Activity Rhythm In Aging And Alzheimers-Disease.* Biological Psychiatry, 1990. **27**(6): p. 563-572.
353. Wagner, D.R., *Disorders of the circadian sleep-wake cycle.* Neurologic Clinics, 1996. **14**(3): p. 651-&.
354. Morgenthaler, T.I., et al., *Practice parameters for the clinical evaluation and treatment of circadian rhythm sleep disorders.* Sleep, 2007. **30**(11): p. 1445-1459.
355. Singer, C., et al., *A multicenter, placebo-controlled trial of melatonin for sleep disturbance in Alzheimer's disease.* Sleep, 2003. **26**(7): p. 893-901.
356. Riemersma-van der Lek, R.F., et al., *Effect of bright light and melatonin on cognitive and noncognitive function in elderly residents of group care facilities - A randomized controlled trial.* Jama-Journal Of The American Medical Association, 2008. **299**(22): p. 2642-2655.
357. Boulos, Z., et al., *Light Treatment for Sleep Disorders: Consensus Report.* Journal of Biological Rhythms, 1995. **10**(2): p. 167-176.
358. Davidson, A.J., et al., *Chronic jet-lag increases mortality in aged mice.* Current Biology, 2006. **16**(21): p. R914-R916.
359. Dijk, D.J., et al., *Ageing and the circadian and homeostatic regulation of human sleep during forced desynchrony of rest, melatonin and temperature rhythms.* Journal Of Physiology-London, 1999. **516**(2): p. 611-627.
360. Reddy, A.B., et al., *Circadian clocks: Neural and peripheral pacemakers that impact upon the cell division cycle.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2005. **574**(1â€“2): p. 76.
361. Cajochen, C., et al., *Evening exposure to blue light stimulates the expression of the clock gene PER2 in humans.* European Journal of Neuroscience, 2006. **23**(4): p. 1082.
362. Filipski, E., et al., *Effects of Chronic Jet Lag on Tumor Progression in Mice.* Cancer Research, 2004. **64**(21): p. 7879-7885.

363. Burgess, H.J., et al., *Preflight adjustment to eastward travel: 3 days of advancing sleep with and without morning bright light.* Journal Of Biological Rhythms, 2003. **18**(4): p. 318-328.
364. Boulos, Z., et al., *Light treatment for sleep disorders: Consensus report.7. Jet lag.* Journal Of Biological Rhythms, 1995. **10**(2): p. 167-176.
365. Akerstedt, T., *Psychological And Psychophysiological Effects Of Shift Work.* Scandinavian Journal Of Work Environment & Health, 1990. **16**: p. 67-73.
366. Boivin, D.B., G.M. Tremblay, and F.O. James, *Working on atypical schedules.* Sleep Medicine, 2007. **8**(6): p. 578-589.
367. Knutsson, A., *Health disorders of shift workers.* Occupational Medicine-Oxford, 2003. **53**(2): p. 103-108.
368. Schernhammer, E.S., et al., *Night-shift work and risk of colorectal cancer in the Nurses' Health Study.* Journal Of The National Cancer Institute, 2003. **95**(11): p. 825-828.
369. Tepas, D.I., et al., *The impact of night work on subjective reports of well-being: an exploratory study of health care workers from five nations.* Revista De Saude Publica, 2004. **38**: p. 26-31.
370. Jorgensen, K.M. and M.D. Witting, *Does exogenous melatonin improve day sleep or night alertness in emergent physicians working night shifts?* Annals Of Emergency Medicine, 1998. **31**(6): p. 699-704.
371. Sharkey, K.M., L.F. Fogg, and C.I. Eastman, *Effects of melatonin administration on daytime sleep after simulated night shift work.* Journal Of Sleep Research, 2001. **10**(3): p. 181-192.
372. Rajaratnam, S.M.W., et al., *Melatonin agonist tasimelteon (VEC-162) for transient insomnia after sleep-time shift: two randomised controlled multicentre trials.* Lancet, 2009. **373**(9662): p. 482-491.
373. Aeschbach, D., et al., *Use of Transdermal Melatonin Delivery to Improve Sleep Maintenance During Daytime.* Clinical Pharmacology & Therapeutics, 2009. **86**(4): p. 378-382.
374. Boivin, D.B., *Influence of sleep-wake and circadian rhythm disturbances in psychiatric disorders.* Journal Of Psychiatry & Neuroscience, 2000. **25**(5): p. 446-458.
375. Bunney, W.E. and B.G. Bunney, *Molecular clock genes in man and lower animals: Possible implications for circadian abnormalities in depression.* Neuropsychopharmacology, 2000. **22**(4): p. 335-345.
376. Benedetti, F., et al., *Influence of CLOCK gene polymorphism on circadian mood fluctuation and illness recurrence in bipolar depression.* American Journal Of Medical Genetics Part B-Neuropsychiatric Genetics, 2003. **123B**(1): p. 23-26.
377. Desan, P.H., et al., *Genetic polymorphism at the CLOCK gene locus and major depression.* American Journal Of Medical Genetics, 2000. **96**(3): p. 418-421.
378. Benedetti, F., et al., *Long-term response to lithium salts in bipolar illness is influenced by the glycogen synthase kinase 3-beta -50 T/C SNP.* Neuroscience Letters, 2005. **376**(1): p. 51-55.
379. Yin, L., et al., *Nuclear receptor Rev-erba is a critical lithium-sensitive component of the circadian clock.* Science, 2006. **311**(5763): p. 1002-1005.
380. Mansour, H.A., T.H. Monk, and V.L. Nimgaonkar, *Circadian genes and bipolar disorder.* Annals Of Medicine, 2005. **37**(3): p. 196-205.

381. Takao, T., et al., *Association of treatment-resistant schizophrenia with the G2677A/T and C3435T polymorphisms in the ATP-binding cassette subfamily B member 1 gene.* Psychiatric Genetics, 2006. **16**(2): p. 47-48 10.1097/01.ypg.0000194441.04684.db.
382. Morton, A.J., et al., *Disintegration of the Sleep-Wake Cycle and Circadian Timing in Huntington's Disease.* The Journal of Neuroscience, 2005. **25**(1): p. 157-163.
383. Dunlap J, L.J., DeCoursey P., *Chronobiology.* Sinauer Associates; Sunderland, MA, 2004.
384. Allada, R. and R.-A. Meissner, *Casein kinase 2, circadian clocks, and the flight from mutagenic light.* Molecular and Cellular Biochemistry, 2005. **274**(1): p. 141.
385. Chang, L. and M. Karin, *Mammalian MAP kinase signalling cascades.* Nature, 2001. **410**(6824): p. 37.
386. Hanahan, D. and R.A. Weinberg, *The Hallmarks of Cancer.* Cell, 2000. **100**(1): p. 57.
387. Lewis, T.S., P.S. Shapiro, and N.G. Ahn, *Signal transduction through MAP kinase cascades.* Advances In Cancer Research, Vol 74, 1998. **74**: p. 49-139.
388. Raman, M. and M.H. Cobb, *MAP Kinase Modules: Many Roads Home.* Current Biology, 2003. **13**(22): p. R886.
389. Ptashne, M. and A. Gann, *Imposing Specificity on Kinases.* Science, 2003. **299**(5609): p. 1025-1027.
390. Ashwell, J.D., *The many paths to p38 mitogen-activated protein kinase activation in the immune system.* Nat Rev Immunol, 2006. **6**(7): p. 532.
391. Coogan, A.N. and H.D. Piggins, *MAP kinases in the mammalian circadian system – key regulators of clock function.* Journal of Neurochemistry, 2004. **90**(4): p. 769.
392. Akashi, M. and E. Nishida, *Involvement of the MAP kinase cascade in resetting of the mammalian circadian clock.* Genes & Development, 2000. **14**(6): p. 645-649.
393. Nakaya, M., K. Sanada, and Y. Fukada, *Spatial and temporal regulation of mitogen-activated protein kinase phosphorylation in the mouse suprachiasmatic nucleus.* Biochemical And Biophysical Research Communications, 2003. **305**(3): p. 494-501.
394. Esquifino, A.I., et al., *Twenty-four-hour rhythms in immune responses in rat submaxillary lymph nodes and spleen: Effect of cyclosporine.* Brain Behavior And Immunity, 1996. **10**(2): p. 92-102.
395. Arjona, A. and D.K. Sarkar, *Circardian oscillations of clock genes, cytolytic factors, and cytokines in rat NK cells.* Journal Of Immunology, 2005. **174**(12): p. 7618-7624.
396. Fernandes, G., et al., *Circadian Rhythmic Plaque-Forming Cell Response Of Spleens From Mice Immunized With Srbc.* Journal Of Immunology, 1976. **117**(3): p. 962-966.
397. Young, M.R.I., et al., *Circadian Rhythmometry Of Serum Interleukin-2, Interleukin-10, Tumor-Necrosis-Factor-Alpha, And Granulocyte-Macrophage Colony-Stimulating Factor In Men.* Chronobiology International, 1995. **12**(1): p. 19-27.
398. Krieger, D.T., *Rhythms Of Acth And Corticosteroid Secretion In Health And Disease, And Their Experimental Modification.* Journal Of Steroid Biochemistry And Molecular Biology, 1975. **6**(5): p. 785-791.
399. Keller, M., et al., *A circadian clock in macrophages controls inflammatory immune responses.* Proceedings of the National Academy of Sciences, 2009. **106**(50): p. 21407-21412.
400. Hunt, T. and P. Sassone-Corsi, *Riding Tandem: Circadian Clocks and the Cell Cycle.* Cell, 2007. **129**(3): p. 461.

401. Matsuo, T., et al., *Control mechanism of the circadian clock for timing of cell division in vivo.* Science, 2003. **302**(5643): p. 255-259.
402. Fu, L.N., et al., *The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo.* Cell, 2002. **111**(1): p. 41-50.
403. You, Z.B., et al., *Wnt signaling promotes oncogenic transformation by inhibiting c-Myc-induced apoptosis.* Journal Of Cell Biology, 2002. **157**(3): p. 429-440.
404. Pucci, B., M. Kasten, and A. Giordano, *Cell cycle and apoptosis.* Neoplasia, 2000. **2**(4): p. 291-299.
405. Evan, G.I. and K.H. Vousden, *Proliferation, cell cycle and apoptosis in cancer.* Nature, 2001. **411**(6835): p. 342-348.
406. Roy, P.G. and A.M. Thompson, *Cyclin D1 and breast cancer.* Breast, 2006. **15**(6): p. 718-727.
407. Smaaland, R., *Circadian rhythm of cell division.* Progress in cell cycle research, 1996. **2**: p. 241-66.
408. Levi, F., et al., *Cross-talks between circadian timing system and cell division cycle determine cancer biology and therapeutics.* Cold Spring Harbor Symposia On Quantitative Biology, 2007. **72**: p. 465-475.
409. van den Heiligenberg, S., et al., *The tumor promoting effect of constant light exposure on diethylnitrosamine-induced hepatocarcinogenesis in rats.* Life Sciences, 1999. **64**(26): p. 2523-2534.
410. Filipski, E., et al., *Disruption of circadian coordination accelerates malignant growth in mice.* Pathologie Biologie, 2003. **51**(4): p. 216.
411. Chen, S.T., et al., *Deregulated expression of the PER1, PER2 and PER3 genes in breast cancers.* Carcinogenesis, 2005. **26**(7): p. 1241-1246.
412. Gery, S., et al., *Transcription profiling of C/EBP targets identifies Per2 as a gene implicated in myeloid leukemia.* Blood, 2005. **106**(8): p. 2827-2836.
413. Gery, S., et al., *The circadian gene Per1 plays an important role in cell growth and DNA damage control in human cancer cells.* Molecular Cell, 2006. **22**(3): p. 375-382.
414. Hoffman, A.E., et al., *The circadian gene NPAS2, a putative tumor suppressor, is involved in DNA damage response.* Molecular Cancer Research, 2008. **6**(9): p. 1461-1468.
415. Dreesen, O. and A.H. Brivanlou, *Signaling pathways in cancer and embryonic stem cells.* Stem Cell Reviews, 2007. **3**(1): p. 7-17.
416. Darnell, J.E., *Transcription factors as targets for cancer therapy.* Nat Rev Cancer, 2002. **2**(10): p. 740.
417. Bromberg, J., *Stat proteins and oncogenesis.* The Journal of Clinical Investigation, 2002. **109**(9): p. 1139.
418. Slamon, D.J., et al., *Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene.* Science, 1987. **235**(4785): p. 177-182.
419. Yarden, Y. and A. Ullrich, *Growth-Factor Receptor Tyrosine Kinases.* Annual Review Of Biochemistry, 1988. **57**: p. 443-478.
420. Darnell, J.E., *Validating Stat3 in cancer therapy.* Nat Med, 2005. **11**(6): p. 595.
421. Roy, M., W.S. Pear, and J.C. Aster, *The multifaceted role of Notch in cancer.* Current Opinion in Genetics & Development, 2007. **17**(1): p. 52.
422. Stylianou, S., R.B. Clarke, and K. Brennan, *Aberrant Activation of Notch Signaling in Human Breast Cancer.* Cancer Research, 2006. **66**(3): p. 1517-1525.

423. Weng, A.P., et al., *Activating Mutations of NOTCH1 in Human T Cell Acute Lymphoblastic Leukemia.* Science, 2004. **306**(5694): p. 269-271.
424. Katz, M., I. Amit, and Y. Yarden, *Regulation of MAPKs by growth factors and receptor tyrosine kinases.* Biochimica Et Biophysica Acta-Molecular Cell Research, 2007. **1773**(8): p. 1161-1176.
425. Sebolt-Leopold, J.S. and R. Herrera, *Targeting the mitogen-activated protein kinase cascade to treat cancer.* Nat Rev Cancer, 2004. **4**(12): p. 937.
426. Vivanco, I. and C.L. Sawyers, *The phosphatidylinositol 3-Kinase-AKT pathway in human cancer.* Nat Rev Cancer, 2002. **2**(7): p. 489.
427. Hennessy, B.T., et al., *Exploiting the PI3K/AKT Pathway for Cancer Drug Discovery.* Nat Rev Drug Discov, 2005. **4**(12): p. 988.
428. Kang, S., A.G. Bader, and P.K. Vogt, *Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(3): p. 802-807.
429. Samuels, Y., et al., *High Frequency of Mutations of the PIK3CA Gene in Human Cancers.* Science, 2004. **304**(5670): p. 554.
430. Woenckhaus, J., et al., *Genomic gain of PIK3CA and increased expression of p110alpha are associated with progression of dysplasia into invasive squamous cell carcinoma.* The Journal of Pathology, 2002. **198**(3): p. 335.
431. Mizoguchi, M., et al., *Genetic alterations of phosphoinositide 3-kinase subunit genes in human glioblastomas.* Brain Pathology, 2004. **14**(4): p. 372-377.
432. Bader, A.G., et al., *Oncogenic PI3K deregulates transcription and translation.* Nat Rev Cancer, 2005. **5**(12): p. 921.
433. Luo, J., B.D. Manning, and L.C. Cantley, *Targeting the PI3K-Akt pathway in human cancer: Rationale and promise.* Cancer Cell, 2003. **4**(4): p. 257.
434. Wullschleger, S., R. Loewith, and M.N. Hall, *TOR Signaling in Growth and Metabolism.* Cell, 2006. **124**(3): p. 471.
435. Brivanlou, A.H. and J.E. Darnell, *Signal Transduction and the Control of Gene Expression.* Science, 2002. **295**(5556): p. 813-818.
436. Courtois, G. and T.D. Gilmore, *Mutations in the NF-[kappa]B signaling pathway: implications for human disease.* Oncogene. **25**(51): p. 6831.
437. Barth, T.F.E., et al., *Gains of 2p involving the REL locus correlate with nuclear c-Rel protein accumulation in neoplastic cells of classical Hodgkin lymphoma.* Blood, 2003. **101**(9): p. 3681-3686.
438. Gilmore, T.D., et al., *Malignant transformation of primary chicken spleen cells by human transcription factor c-Rel.* Oncogene, 2001. **20**(48): p. 7098-7103.
439. Logan, C.Y. and R. Nusse, *The Wnt signaling pathway in development and disease.* Annual Review Of Cell And Developmental Biology, 2004. **20**: p. 781-810.
440. Tetsu, O. and F. McCormick, *[beta]-Catenin regulates expression of cyclin D1 in colon carcinoma cells.* Nature, 1999. **398**(6726): p. 422.
441. Giles, R.H., J.H. van Es, and H. Clevers, *Caught up in a Wnt storm: Wnt signaling in cancer.* Biochimica Et Biophysica Acta-Reviews On Cancer, 2003. **1653**(1): p. 1-24.
442. Bardeesy, N., et al., *Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer.* Genes & Development, 2006. **20**(22): p. 3130-3146.

443. Langenfeld, E.M., Y. Kong, and J. Langenfeld, *Bone morphogenetic protein 2 stimulation of tumor growth involves the activation of Smad-1//5.* Oncogene, 2005. **25**(5): p. 685.
444. Turek, F.W., et al., *Obesity and Metabolic Syndrome in Circadian Clock Mutant Mice.* Science, 2005. **308**(5724): p. 1043-1045.
445. Rudic, R.D., et al., *BMAL1 and CLOCK, Two Essential Components of the Circadian Clock, Are Involved in Glucose Homeostasis.* PLoS Biol, 2004. **2**(11): p. e377.
446. Oishi, K., et al., *Genome-wide Expression Analysis of Mouse Liver Reveals CLOCK-regulated Circadian Output Genes.* Journal of Biological Chemistry, 2003. **278**(42): p. 41519-41527.
447. Brooks, C.L. and W. Gu, *How does SIRT1 affect metabolism, senescence and cancer?* Nat Rev Cancer, 2009. **9**(2): p. 123.
448. Vaziri, H., et al., *hSIR2SIRT1 Functions as an NAD-Dependent p53 Deacetylase.* Cell, 2001. **107**(2): p. 149.
449. Cohen, H.Y., et al., *Acetylation of the C Terminus of Ku70 by CBP and PCAF Controls Bax-Mediated Apoptosis.* Molecular Cell, 2004. **13**(5): p. 627.
450. Wang, R.-H., et al., *Impaired DNA Damage Response, Genome Instability, and Tumorigenesis in SIRT1 Mutant Mice.* Cancer Cell, 2008. **14**(4): p. 312.
451. Firestein, R., et al., *The SIRT1 Deacetylase Suppresses Intestinal Tumorigenesis and Colon Cancer Growth.* PLoS ONE, 2008. **3**(4): p. e2020.
452. Garten, A., et al., *Nampt: linking NAD biology, metabolism and cancer.* Trends in Endocrinology & Metabolism, 2009. **20**(3): p. 130.
453. Zhang, R. and X. Xie, *Tools for GPCR drug discovery.* Acta Pharmacologica Sin.
454. Pires-daSilva, A. and R.J. Sommer, *The evolution of signalling pathways in animal development.* Nat Rev Genet, 2003. **4**(1): p. 39.
455. Levy, S., et al., *The Diploid Genome Sequence of an Individual Human.* PLoS Biol, 2007. **5**(10): p. e254.
456. Evans, W.E. and J.A. Johnson, *PHARMACOGENOMICS: The Inherited Basis for Interindividual Differences in Drug Response.* Annual Review of Genomics and Human Genetics, 2001. **2**(1): p. 9.
457. Evans, W.E. and H.L. McLeod, *Pharmacogenomics â€" Drug Disposition, Drug Targets, and Side Effects.* New England Journal of Medicine, 2003. **348**(6): p. 538-549.
458. Theuns, J., et al., *Genetic variability in the regulatory region of presenilin 1 associated with risk for Alzheimerâ€™s disease and variable expression.* Human Molecular Genetics, 2000. **9**(3): p. 325-331.
459. Todd, J.A., et al., *Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes.* Nat Genet, 2007. **39**(7): p. 857.
460. Hakonarson, H., et al., *A genome-wide association study identifies KIAA0350 as a type 1 diabetes gene.* Nature, 2007. **448**(7153): p. 591.
461. Sladek, R., et al., *A genome-wide association study identifies novel risk loci for type 2 diabetes.* Nature, 2007. **445**(7130): p. 881.
462. Scott, L.J., et al., *A Genome-Wide Association Study of Type 2 Diabetes in Finns Detects Multiple Susceptibility Variants.* Science, 2007. **316**(5829): p. 1341-1345.
463. Forsell, P.A.L., et al., *Genomic characterization of the human and mouse protein tyrosine phosphatase-1B genes.* Gene, 2000. **260**(1â€“2): p. 145.

464. Karp, C.L., et al., *Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma.* Nature Immunology, 2000. **1**(3): p. 221-226.
465. Miki, Y., et al., *A Strong Candidate For The Breast And Ovarian-Cancer Susceptibility Gene Brca1.* Science, 1994. **266**(5182): p. 66-71.
466. Wooster, R., et al., *Identification of the breast cancer susceptibility gene BRCA2.* Nature, 1995. **378**(6559): p. 789.
467. Cormier, R.T., et al., *Secretory phospholipase Pla2g2a confers resistance to intestinal tumorigenesis.* Nature Genetics, 1997. **17**(1): p. 88-91.
468. Carl, B., et al., *B-lymphoblastoid cell lines as a source of reference DNA for human platelet and neutrophil antigen genotyping.* Transfusion, 2000. **40**(1): p. 62.
469. Cheung, V.G., et al., *Mapping determinants of human gene expression by regional and genome-wide association.* Nature, 2005. **437**(7063): p. 1365.
470. Stranger, B.E., et al., *Relative Impact of Nucleotide and Copy Number Variation on Gene Expression Phenotypes.* Science, 2007. **315**(5813): p. 848-853.
471. Veyrieras, J.-B., et al., *High-Resolution Mapping of Expression-QTLs Yields Insight into Human Gene Regulation.* PLoS Genet, 2008. **4**(10): p. e1000214.
472. Stranger, B.E., et al., *Population genomics of human gene expression.* Nat Genet, 2007. **39**(10): p. 1217.
473. Dixon, A.L., et al., *A genome-wide association study of global gene expression.* Nat Genet, 2007. **39**(10): p. 1202.
474. Badhai, J., et al., *Posttranscriptional down-regulation of small ribosomal subunit proteins correlates with reduction of 18S rRNA in RPS19 deficiency.* Febs Letters, 2009. **583**(12): p. 2049-2053.
475. Correa, C.R. and V.G. Cheung, *Genetic variation in radiation-induced expression phenotypes.* American Journal Of Human Genetics, 2004. **75**(5): p. 885-890.
476. Niu, N., et al., *Radiation pharmacogenomics: A genome-wide association approach to identify radiation response biomarkers using human lymphoblastoid cell lines.* Genome Research. **20**(11): p. 1482-1492.
477. Huang, R.S., et al., *Identification of genetic variants contributing to cisplatin-induced cytotoxicity by use of a genomewide approach.* American Journal Of Human Genetics, 2007. **81**(3): p. 427-437.
478. Duan, S., et al., *Mapping genes that contribute to daunorubicin-induced cytotoxicity.* Cancer Research, 2007. **67**(11): p. 5425-5433.
479. Wheeler, H.E. and M.E. Dolan, *Lymphoblastoid cell lines in pharmacogenomic discovery and clinical translation.* Pharmacogenomics. **13**(1): p. 55-70.
480. Zhou, S.-F., J.-P. Liu, and B. Chowbay, *Polymorphism of human cytochrome P450 enzymes and its clinical impact.* Drug Metabolism Reviews, 2009. **41**(2): p. 89-295.
481. Kiyotani, K., et al., *Decreased coumarin 7-hydroxylase activities and CYP2A6 expression levels in humans caused by genetic polymorphism in CYP2A6 promoter region (CYP2A6*9).* Pharmacogenetics and Genomics, 2003. **13**(11): p. 689-695.
482. Ross, D.T., et al., *Systematic variation in gene expression patterns in human cancer cell lines.* Nat Genet, 2000. **24**(3): p. 227.
483. Scherf, U., et al., *A gene expression database for the molecular pharmacology of cancer.* Nat Genet, 2000. **24**(3): p. 236.
484. Garraway, L.A., et al., *Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma.* Nature, 2005. **436**(7047): p. 117.

485. Nishizuka, S., et al., *Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays.* Proceedings of the National Academy of Sciences, 2003. **100**(24): p. 14229-14234.
486. Weinstein, J.N., et al., *An Information-Intensive Approach to the Molecular Pharmacology of Cancer.* Science, 1997. **275**(5298): p. 343-349.
487. Dai, Z.Y., et al., *Prediction of anticancer drug potency from expression of genes involved in growth factor signaling.* Pharmaceutical Research, 2006. **23**(2): p. 336-349.
488. Shankavaram, U.T., et al., *Transcript and protein expression profiles of the NCI-60 cancer cell panel: an integromic microarray study.* Molecular Cancer Therapeutics, 2007. **6**(3): p. 820-832.
489. Staunton, J.E., et al., *Chemosensitivity prediction by transcriptional profiling.* Proceedings of the National Academy of Sciences, 2001. **98**(19): p. 10787-10792.
490. Le Morvan, V.r., et al., *Relationships between genetic polymorphisms and anticancer drug cytotoxicity vis-Ã -vis the NCI-60 panel.* Pharmacogenomics, 2006. **7**(6): p. 843.
491. Puyo, S.p., V.r. Le Morvan, and J. Robert, *Impact of EGFR Gene Polymorphisms on Anticancer Drug Cytotoxicity In Vitro.* Molecular Diagnosis & Therapy, 2008. **12**(4): p. 225-234 10.2165/1250444-200812040-00004.
492. Sasaki, S., et al., *DNA methylation and sensitivity to antimetabolites in cancer cell lines.* Oncology Reports, 2008. **19**(2): p. 407-412.
493. Zhang, W., et al., *Genome-wide identification of genetic determinants for the cytotoxicity of perifosine.* Human Genomics, 2008. **3**(1): p. 53-70.
494. Takahashi, K. and S. Yamanaka, *Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors.* Cell, 2006. **126**(4): p. 663.
495. Yu, J., et al., *Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells.* Science, 2007. **318**(5858): p. 1917-1920.
496. Lowry, W.E., et al., *Generation of human induced pluripotent stem cells from dermal fibroblasts.* Proceedings of the National Academy of Sciences, 2008. **105**(8): p. 2883-2888.
497. Takahashi, K., et al., *Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors.* Cell, 2007. **131**(5): p. 861.
498. Urbach, A., et al., *Differential Modeling of Fragile X Syndrome by Human Embryonic Stem Cells and Induced Pluripotent Stem Cells.* Cell Stem Cell. **6**(5): p. 407.
499. Lee, G., et al., *Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs.* Nature, 2009. **461**(7262): p. 402.
500. Carvajal-Vergara, X., et al., *Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome.* Nature. **465**(7299): p. 808.
501. Zhang, J., et al., *A Human iPSC Model of Hutchinson Gilford Progeria Reveals Vascular Smooth Muscle and Mesenchymal Stem Cell Defects.* Cell Stem Cell. **8**(1): p. 31.
502. Rashid, S.T., et al., *Modeling inherited metabolic disorders of the liver using human induced pluripotent stem cells.* Journal Of Clinical Investigation. **120**(9): p. 3127-3136.
503. Ebert, A.D., et al., *Induced pluripotent stem cells from a spinal muscular atrophy patient.* Nature, 2009. **457**(7227): p. 277.
504. Levy, S., et al., *The diploid genome sequence of an individual human.* PLoS biology, 2007. **5**(10): p. e254.

505. Lowrey, P.L. and J.S. Takahashi, *MAMMALIAN CIRCADIAN BIOLOGY: Elucidating Genome-Wide Levels of Temporal Organization.* Annual Review of Genomics and Human Genetics, 2004. **5**(1): p. 407.
506. Horne, J.A. and O. Ostberg, *A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms.* International journal of chronobiology, 1976. **4**(2): p. 97-110.
507. Almirall, H., *INCLUDING NEITHER-TYPE IN THE MORNINGNESS-EVENINGNESS DIMENSION DECREASES THE ROBUSTNESS OF THE MODEL.* Perceptual and Motor Skills, 1993. **77**(1): p. 243.
508. Carrier, J., et al., *Sleep and morningness-eveningness in the 'middle' years of life (20–59y).* Journal of Sleep Research, 1997. **6**(4): p. 230.
509. Czeisler, C.A., et al., *Association of sleep-wake habits in older people with changes in output of circadian pacemaker.* The Lancet, 1992. **340**(8825): p. 933.
510. Monk, T.H. and V.C. Leng, *Interactions Between Inter-Individual And Inter-Task Differences In The Diurnal Variation Of Human Performance.* Chronobiology International, 1986. **3**(3): p. 171-178.
511. Motohashi, Y.H., S.; Maeda, A., *Men's Time, Women's Time Sex Differences in Biological Time Structure.* Appl. Human Sci, 1998. **17**(4): p. 157-159.
512. Heath, A.C., et al., *Evidence For Genetic Influences On Sleep Disturbance And Sleep Pattern In Twins.* Sleep, 1990. **13**(4): p. 318-335.
513. Vink, J.M., et al., *GENETIC ANALYSIS OF MORNINGNESS AND EVENINGNESS.* Chronobiology International, 2001. **18**(5): p. 809.
514. Klei, L., et al., *Heritability of Morningnessâ€□Eveningness and Selfâ€□Report Sleep Measures in a Familyâ€•Based Sample of 521 Hutterites.* Chronobiology International, 2005. **22**(6): p. 1041.
515. Koskenvuo, M., et al., *Heritability of diurnal type: a nationwide study of 8753 adult twin pairs.* Journal of Sleep Research, 2007. **16**(2): p. 156.
516. Mishima, K., et al., *The 3111T/C polymorphism of hClock is associated with evening preference and delayed sleep timing in a Japanese population sample.* American Journal Of Medical Genetics Part B-Neuropsychiatric Genetics, 2005. **133B**(1): p. 101-104.
517. Carpen, J.D., et al., *A single-nucleotide polymorphism in the 5 '-untranslated region of the hPER2 gene is associated with diurnal preference.* Journal Of Sleep Research, 2005. **14**(3): p. 293-297.
518. Fu, Y., et al., *An hPer2 phosphorylation site mutation in familial Advanced Sleep-Phase Syndrome.* American Journal Of Human Genetics, 2001. **69**(4): p. 597-597.
519. Toh, K.L., et al., *An hPer2 Phosphorylation Site Mutation in Familial Advanced Sleep Phase Syndrome.* Science, 2001. **291**(5506): p. 1040-1043.
520. Satoh, K., et al., *Two pedigrees of familial advanced sleep phase syndrome in Japan.* Sleep, 2003. **26**(4): p. 416-417.
521. Johansson, C., et al., *Circadian clock-related polymorphisms in seasonal affective disorder and their relevance to diurnal preference.* Neuropsychopharmacology, 2003. **28**(4): p. 734-739.
522. Archer, S.N., et al., *A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference.* Sleep, 2003. **26**: p. A109-A109.

523. Carpen, J., et al., *A silent polymorphism in the <i>PER1</i> gene associates with extreme diurnal preference in humans.* Journal of Human Genetics, 2006. **51**(12): p. 1122.
524. Yamazaki, S., et al., *Resetting Central and Peripheral Circadian Oscillators in Transgenic Rats.* Science, 2000. **288**(5466): p. 682-685.
525. Loeuillet, C., et al., *In Vitro Whole-Genome Analysis Identifies a Susceptibility Locus for HIV-1.* PLoS Biol, 2008. **6**(2): p. e32.
526. Akashi, M., et al., *Noninvasive method for assessing the human circadian clock using hair follicle cells.* Proceedings of the National Academy of Sciences. **107**(35): p. 15643-15648.
527. Boivin, D.B., et al., *Circadian clock genes oscillate in human peripheral blood mononuclear cells.* Blood, 2003. **102**(12): p. 4143-4145.
528. Kusanagi, H., et al., *Similar profiles in human period1 gene expression in peripheral mononuclear and polymorphonuclear cells.* Neuroscience Letters, 2004. **365**(2): p. 124-127.
529. Takimoto, M., et al., *Daily expression of clock genes in whole blood cells in healthy subjects and a patient with circadian rhythm sleep disorder.* American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 2005. **289**(5): p. R1273-R1279.
530. Teboul, M., et al., *Atypical patterns of circadian clock gene expression in human peripheral blood mononuclear cells.* Journal of Molecular Medicine, 2005. **83**(9): p. 693.
531. Fukuya, H., et al., *Circadian expression of clock genes in human peripheral leukocytes.* Biochemical And Biophysical Research Communications, 2007. **354**(4): p. 924-928.
532. Arjona, A. and D.K. Sarkar, *Evidence supporting a circadian control of natural killer cell function.* Brain, Behavior, and Immunity, 2006. **20**(5): p. 469.
533. Arjona, A. and D.K. Sarkar, *Circadian Oscillations of Clock Genes, Cytolytic Factors, and Cytokines in Rat NK Cells.* The Journal of Immunology, 2005. **174**(12): p. 7618-7624.
534. Silver, A.C., et al., *Circadian expression of clock genes in mouse macrophages, dendritic cells, and B cells.* Brain, Behavior, and Immunity. **26**(3): p. 407.
535. Salmon, P. and D. Trono, *Production and Titration of Lentiviral Vectors*, in *Current Protocols in Human Genetics*. 2001, John Wiley & Sons, Inc.
536. Mohyuddin, A., et al., *Genetic instability in EBV-transformed lymphoblastoid cell lines.* Biochimica et Biophysica Acta (BBA) - General Subjects, 2004. **1670**(1): p. 81.
537. Brand, K., et al., *Metabolic Alterations Associated With Proliferation Of Mitogen-Activated Lymphocytes And Of Lymphoblastoid Cell-Lines - Evaluation Of Glucose And Glutamine-Metabolism.* Immunobiology, 1986. **173**(1): p. 23-34.
538. Pando, M.P., et al., *Phenotypic Rescue of a Peripheral Clock Genetic Defect via SCN Hierarchical Dominance.* Cell, 2002. **110**(1): p. 107.
539. Cepko, C., *Large-Scale Preparation and Concentration of Retrovirus Stocks.* Current Protocols in Molecular Biology, 2001(Unit 9.12).
540. Fraefel, C., et al., *Helper virus-free transfer of herpes simplex virus type 1 plasmid vectors into neural cells.* Journal of Virology, 1996. **70**(10): p. 7190-7.
541. Xie, W.Q. and L.I. Rothblum, *Rapid, small-scale RNA isolation from tissue culture cells.* Biotechniques, 1991. **11**(3): p. 324, 326-7.

542. Qiu, X., et al., *Induction of photosensitivity by heterologous expression of melanopsin.* Nature, 2005. **433**(7027): p. 745.
543. Lavery, D.J. and U. Schibler, *Circadian Transcription Of The Cholesterol 7-Alpha Hydroxylase Gene May Involve The Liver-Enriched Bzip Protein Dbp.* Genes & Development, 1993. **7**(10): p. 1871-1884.
544. Rabinowitz, L., *Aldosterone and potassium homeostasis.* Kidney International, 1996. **49**(6): p. 1738-1742.
545. Michael H, H., *Circadian clocks.* Current Biology, 1997. **7**(11): p. R670.
546. Rusak, B. and I. Zucker, *Neural Regulation Of Circadian-Rhythms.* Physiological Reviews, 1979. **59**(3): p. 449-526.
547. Joseph S, T., *Circadian-clock regulation of gene expression.* Current Opinion in Genetics & Development, 1993. **3**(2): p. 301.
548. Yamazaki, S., et al., *Resetting Central and Peripheral Circadian Oscillators in Transgenic Rats.* Science, 2000. **288**(5466): p. 682.
549. Balsalobre, A.l., F. Damiola, and U. Schibler, *A Serum Shock Induces Circadian Gene Expression in Mammalian Tissue Culture Cells.* Cell, 1998. **93**(6): p. 929.
550. Welsh, D.K., J.S. Takahashi, and S.A. Kay, *Suprachiasmatic Nucleus: Cell Autonomy and Network Properties*, in *Annual Review Of Physiology*. p. 551-577.
551. Brown, S.A., et al., *Molecular insights into human daily behavior.* Proceedings of the National Academy of Sciences, 2008. **105**(5): p. 1602-1607.
552. Hunter, D.J., et al., *A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer.* Nat Genet, 2007. **39**(7): p. 870.
553. Zeggini, E. and M.I. McCarthy, *Identifying susceptibility variants for type 2 diabetes.* Methods in molecular biology (Clifton, N.J.), 2007. **376**: p. 235-50.
554. Mayer, T., et al., *Cellâ€•Based Assays Using Primary Endothelial Cells to Study Multiple Steps in Inflammation*, in *Methods in Enzymology*. 2006, Academic Press. p. 266.
555. Wyler, M.R., et al., *Cell-Based Assays to Probe the ERK MAP Kinase Pathway in Endothelial Cells*. 2008. p. 29-41.
556. Tiscornia, G., E.L. Vivas, and J.C.I. Belmonte, *Diseases in a dish: modeling human genetic disorders using induced pluripotent cells.* Nat Med: p. 1570.
557. Naldini, L., et al., *Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 1996. **93**(21): p. 11382-11388.
558. Reiser, J., et al., *Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 1996. **93**(26): p. 15266-15271.
559. Case, S.S., et al., *Stable transduction of quiescent CD34(+)CD38(-) human hematopoietic cells by HIV-1-based lentiviral vectors.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 1999. **96**(6): p. 2988-2993.
560. R K Akkina, R.M.W., M L Chen, Q X Li, V Planelles and I S Chen, *High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G.* J. Virol. **70**: p. 2581-5.

561. Naldini, L., et al., *In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector.* Science, 1996. **272**(5259): p. 263-267.
562. Tal Kafri, U.B., Daniel A. Peterson, Fred H. Gage & Inder M. Verma, *Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors.* Nature Genetics, 1997(17): p. 314 - 317.
563. Brown, S.A., et al., *The Period Length of Fibroblast Circadian Gene Expression Varies Widely among Human Individuals.* PLoS Biol, 2005. **3**(10): p. e338.
564. Pagani, L., et al., *The Physiological Period Length of the Human Circadian Clock <italic>In Vivo</italic> Is Directly Proportional to Period in Human Fibroblasts.* PLoS ONE. **5**(10): p. e13376.
565. Zhang, X.M., et al., *Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 2005. **102**(12): p. 4459-4464.
566. Boros, J., et al., *Overlapping promoter targeting by Elk-1 and other divergent ETS-domain transcription factor family members.* Nucleic Acids Research, 2009. **37**(22): p. 7368-7380.
567. Wang, X.-Z., et al., *Identification of novel stress-induced genes downstream of chop.* EMBO J, 1998. **17**(13): p. 3619.
568. A J Fornace, J., I Alamo, Jr, and M C Hollander, *DNA damage-inducible transcripts in mammalian cells.* PNAS, 1988. **85**(23): p. 8800-8804.
569. Vanecek, J., et al., *Atypical Synergistic Alpha-1-Adrenergic And Beta-Adrenergic Regulation Of Adenosine-3',5'-Monophosphate And Guanosine 3',5'-Monophosphate In Rat Pinealocytes.* Endocrinology, 1985. **116**(6): p. 2167-2173.
570. Roseboom, P.H. and D.C. Klein, *Norepinephrine Stimulation Of Pineal Cyclic-Amp Response Element-Binding Protein-Phosphorylation - Primary Role Of A Beta-Adrenergic-Receptor Cyclic-Amp Mechanism.* Molecular Pharmacology, 1995. **47**(3): p. 439-449.
571. Baler, R., S. Covington, and D.C. Klein, *The rat arylalkylamine N-acetyltransferase gene promoter - cAMP activation via a cAMP-responsive element-CCAAT complex.* Journal Of Biological Chemistry, 1997. **272**(11): p. 6979-6985.
572. Klein, D.C., et al., *Pineal serotonin N-acetyltransferase activity: abrupt decrease in adenosine 3',5'-monophosphate may be signal for "turnoff".* Science, 1978. **199**(4326): p. 309-311.
573. Klein, D.C. and J.L. Weller, *Rapid Light-Induced Decrease in Pineal Serotonin N-Acetyltransferase Activity.* Science, 1972. **177**(4048): p. 532-533.
574. Martoglio, A.M., et al., *Changes in tumorigenesis- and angiogenesis-related gene transcript abundance profiles in ovarian cancer detected by tailored high density cDNA arrays.* Molecular Medicine, 2000. **6**(9): p. 750-765.
575. Andrew Clayton, T., et al., *Pharmaco-metabonomic phenotyping and personalized drug treatment.* Nature, 2006. **440**(7087): p. 1073.
576. Emilsson, V., et al., *Genetics of gene expression and its effect on disease.* Nature, 2008. **452**(7186): p. 423.
577. Dimas, A.S., et al., *Common Regulatory Variation Impacts Gene Expression in a Cell Typeâ€“Dependent Manner.* Science, 2009. **325**(5945): p. 1246-1250.

578. Thompson, C., D. Stinson, and A. Smith, *Seasonal affective disorder and season-dependent abnormalities of melatonin suppression by light.* The Lancet, 1990. **336**(8717): p. 703.
579. Gordijn, M.C.M., et al., *Effects of light exposure and sleep displacement on dim light melatonin onset.* Journal Of Sleep Research, 1999. **8**(3): p. 163-174.
580. Allen, D.D., et al., *Cell Lines as In Vitro Models for Drug Screening and Toxicity Studies.* Drug Development and Industrial Pharmacy, 2005. **31**(8): p. 757.
581. Inoue, H. and S. Yamanaka, *The Use of Induced Pluripotent Stem Cells in Drug Development.* Clin Pharmacol Ther. **89**(5): p. 655.
582. Evans, W.E. and M.V. Relling, *Pharmacogenomics: Translating Functional Genomics into Rational Therapeutics.* Science, 1999. **286**(5439): p. 487-491.
583. Robinton, D.A. and G.Q. Daley, *The promise of induced pluripotent stem cells in research and therapy.* Nature. **481**(7381): p. 295.
584. Ieda, M., et al., *Direct Reprogramming of Fibroblasts into Functional Cardiomyocytes by Defined Factors.* Cell. **142**(3): p. 375.
585. Grskovic, M., et al., *Induced pluripotent stem cells â€" opportunities for disease modelling and drug discovery.* Nat Rev Drug Discov. **10**(12): p. 915.
586. Cepko, C., *Large-scale preparation and concentration of retroviral stocks.* Current protocols in molecular biology, 2001. **3**: p. Chapter 16.
587. Strober, W., *Trypan Blue Exclusion Test of Cell Viability.* Current Protocols in Immunology, 2001: p. Appendix 3B.
588. Lyons, S.K., *Advances in imaging mouse tumour models in vivo.* The Journal of Pathology, 2005. **205**(2): p. 194.
589. Thompson, J.F., L.S. Hayes, and D.B. Lloyd, *Modulation of firefly luciferase stability and impact on studies of gene regulation.* Gene, 1991. **103**(2): p. 171.
590. Cao, F., et al., *In vivo visualization of embryonic stem cell survival, proliferation, and migration after cardiac delivery.* Circulation, 2006. **113**(7): p. 1005-1014.
591. Sacco, A., et al., *Self-renewal and expansion of single transplanted muscle stem cells.* Nature, 2008. **456**(7221): p. 502-506.
592. Toegel, F., et al., *Bioluminescence imaging to monitor the in vivo distribution of administered mesenchymal stem cells in acute kidney injury.* American Journal Of Physiology-Renal Physiology, 2008. **295**(1): p. F315-F321.
593. Dobrenkov, K., et al., *Monitoring the efficacy of adoptively transferred prostate cancer-targeted human T lymphocytes with PET and bioluminescence imaging.* Journal Of Nuclear Medicine, 2008. **49**(7): p. 1162-1170.
594. Chen, X., et al., *In vivo bioluminescence imaging of transplanted islets and early detection of graft rejection.* Transplantation, 2006. **81**(10): p. 1421-1427.
595. Shachaf, C.M., et al., *MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer.* Nature, 2004. **431**(7012): p. 1112-1117.
596. Liu, A.C., et al., *Intercellular coupling confers robustness against mutations in the SCN circadian clock network.* Cell, 2007. **129**(3): p. 605-616.
597. Contag, C.H., et al., *Visualizing gene expression in living mammals using a bioluminescent reporter.* Photochemistry And Photobiology, 1997. **66**(4): p. 523-531.
598. Lehmann, S., et al., *Longitudinal and multimodal in vivo imaging of tumor hypoxia and its downstream molecular events.* Proceedings of the National Academy of Sciences, 2009.

599. Harada, H., et al., *Significance of HIF-1-active cells in angiogenesis and radioresistance.* Oncogene, 2007. **26**(54): p. 7508.
600. Korpal, M., et al., *Imaging transforming growth factor-[beta] signaling dynamics and therapeutic response in breast cancer bone metastasis.* Nat Med, 2009. **15**(8): p. 960.
601. TamgÃ¼ney, G.l., et al., *Measuring prions by bioluminescence imaging.* Proceedings of the National Academy of Sciences, 2009. **106**(35): p. 15002-15006.
602. Rettig, G.R., et al., *Quantitative bioluminescence imaging of transgene expression in vivo.* Analytical Biochemistry, 2006. **355**(1): p. 90.
603. Lamfers, M.L.M., et al., *Cyclophosphamide increases transgene expression mediated by an oncolytic adenovirus in glioma-bearing mice monitored by bioluminescence imaging.* Molecular Therapy, 2006. **14**(6): p. 779-788.
604. Bartlett, D.W. and M.E. Davis, *Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging.* Nucleic Acids Research, 2006. **34**(1): p. 322-333.
605. Luker, G.D., et al., *Imaging 26S proteasome activity and inhibition in living mice.* Nature Medicine, 2003. **9**(7): p. 969-973.
606. Luker, K.E., M. Gupta, and G.D. Luker, *Imaging CXCR4 Signaling with Firefly Luciferase Complementation.* Analytical Chemistry, 2008. **80**(14): p. 5565.
607. Huang, E., et al., *Gene expression phenotypic models that predict the activity of oncogenic pathways.* Nat Genet, 2003. **34**(2): p. 226.
608. Segal, E., et al., *A module map showing conditional activity of expression modules in cancer*. Nat Genet, 2004. **36**(10): p. 1090.
609. Ramaswamy, S. and T.R. Golub, *DNA Microarrays in Clinical Oncology*. Journal of Clinical Oncology, 2002. **20**(7): p. 1932-1941.
610. Wendt, M.K., et al., *In vivo dual substrate bioluminescent imaging.* Journal of visualized experiments: JoVE, (56).
611. Miyazaki, K., et al., *Tumor growth suppression in vivo by overexpression of the circadian component, PER2.* Genes to Cells. **15**(4): p. 351.
612. Xiaoming, Y., et al., *Circadian Time-Dependent Tumor Suppressor Function of Period Genes.* Integrative Cancer Therapies, 2009. **8**(4): p. 309-316.
613. Canaple, L., T. Kakizawa, and V. Laudet, *The Days and Nights of Cancer Cells.* Cancer Research, 2003. **63**(22): p. 7545-7552.
614. Filipski, E., et al., *Host circadian clock as a control point in tumor progression.* Journal Of The National Cancer Institute, 2002. **94**(9): p. 690-697.
615. Gery, S., et al., *The clock gene Per2 links the circadian system to the estrogen receptor.* Oncogene, 2007. **26**(57): p. 7916-7920.
616. Hua, H., et al., *Inhibition of tumorigenesis by intratumoral delivery of the circadian gene mPer2 in C57BL/6 mice.* Cancer Gene Therapy, 2007. **14**(9): p. 815-818.
617. Hua, H., et al., *Circadian gene mPer2 overexpression induces cancer cell apoptosis.* Cancer Science, 2006. **97**(7): p. 589-596.
618. Halberg, F., et al., *Toward a chronotherapy of neoplasia: Tolerance of treatment depends upon host rhythms.* Cellular and Molecular Life Sciences, 1973. **29**(8): p. 909.
619. Mormont, M.-C. and F. Levi, *Cancer chronotherapy: Principles, applications, and perspectives.* Cancer, 2003. **97**(1): p. 155.
620. Blagosklonny, M.V. and A.B. Pardee, *Exploiting cancer cell cycling for selective protection of normal cells.* Cancer Research, 2001. **61**(11): p. 4301-4305.

621. Cecon, E., et al., *Daily Variation Of Constitutively Activated Nuclear Factor Kappa B (Nfkb) In Rat Pineal Gland.* Chronobiology International. **27**(1): p. 52-67.
622. Monks, S.A., et al., *Genetic Inheritance of Gene Expression in Human Cell Lines.* The American Journal of Human Genetics, 2004. **75**(6): p. 1094.
623. Gachon, F., et al., *The mammalian circadian timing system: from gene expression to physiology.* Chromosoma, 2004. **113**(3): p. 103.
624. Yoo, S.H., et al., *PERIOD2: LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 2004. **101**(15): p. 5339-5346.
625. Welsh, D.K., J.S. Takahashi, and S.A. Kay, *Suprachiasmatic Nucleus: Cell Autonomy and Network Properties.* Annual Review of Physiology. **72**(1): p. 551-577.
626. Hughes, M.E., et al., *Harmonics of Circadian Gene Transcription in Mammals.* Plos Genetics, 2009. **5**(4).
627. Gould, S.J. and S. Subramani, *Firefly luciferase as a tool in molecular and cell biology.* Analytical Biochemistry, 1988. **175**(1): p. 5.
628. Alam, J. and J.L. Cook, *Reporter genes: Application to the study of mammalian gene transcription.* Analytical Biochemistry, 1990. **188**(2): p. 245.
629. Williams, T.M., et al., *Advantages of firefly luciferase as a reporter gene: Application to the interleukin-2 gene promoter.* Analytical Biochemistry, 1989. **176**(1): p. 28.
630. Boussif, O., et al., *A Versatile Vector For Gene And Oligonucleotide Transfer Into Cells In Culture And In-Vivo - Polyethylenimine.* Proceedings Of The National Academy Of Sciences Of The United States Of America, 1995. **92**(16): p. 7297-7301.
631. Capecchi, M.R., *High efficiency transformation by direct microinjection of DNA into cultured mammalian cells.* Cell, 1980. **22**(2, Part 2): p. 479.
632. Kawai, S. and M. Nishizawa, *New Procedure For Dna Transfection With Polycation And Dimethylsulfoxide.* Molecular And Cellular Biology, 1984. **4**(6): p. 1172-1174.
633. Kukowska-Latallo, J.F., et al., *Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers.* Proceedings of the National Academy of Sciences, 1996. **93**(10): p. 4897-4902.
634. Morgan, W.F. and J.P. Day, *The Introduction of Proteins into Mammalian Cells by Electroporation*. 1995. p. 63-71.
635. Ogura, R., et al., *Multi-color luciferases as reporter for monitoring regulated gene expression in higher plants.* Plant And Cell Physiology, 2007. **48**: p. S1-S1.
636. Anson, D., *The use of retroviral vectors for gene therapy-what are the risks? A review of retroviral pathogenesis and its relevance to retroviral vector-mediated gene delivery.* Genetic Vaccines and Therapy, 2004. **2**(1): p. 9.
637. Pope, J.H., *Establishment of Cell Lines from Peripheral Leucocytes in Infectious Mononucleosis.* Nature, 1967. **216**(5117): p. 810.
638. Bishop, G.A. and L.K. Busch, *Molecular mechanisms of B-lymphocyte transformation by Epstein-Barr virus.* Microbes And Infection, 2002. **4**(8): p. 853-857.
639. Thompson, M.P. and R. Kurzrock, *Epstein-Barr Virus and Cancer.* Clinical Cancer Research, 2004. **10**(3): p. 803-821.
640. Maruo, S., et al., *Epstein-Barr virus nuclear antigens 3C and 3A maintain lymphoblastoid cell growth by repressing p16INK4A and p14ARF expression.* Proceedings of the National Academy of Sciences. **108**(5): p. 1919-1924.

641. Chen, S.-T., et al., *Deregulated expression of the PER1, PER2 and PER3 genes in breast cancers.* Carcinogenesis, 2005. **26**(7): p. 1241-1246.
642. Satoh, T., M. Fukuda, and T. Sairenji, *Distinct patterns of mitogen-activated protein kinase phosphorylation and Epstein-Barr virus gene expression in Burkitt's lymphoma cell lines versus B lymphoblastoid cell lines.* Virus Genes, 2002. **25**(1): p. 15-21.
643. Guido, M.E., et al., *Circadian and photic regulation of immediate-early gene expression in the hamster suprachiasmatic nucleus.* Neuroscience, 1999. **90**(2): p. 555.
644. Maywood, E.S., J.A. O'Brien, and M.H. Hastings, *Expression of mCLOCK and Other Circadian Clock-Relevant Proteins in the Mouse Suprachiasmatic Nuclei.* Journal of Neuroendocrinology, 2003. **15**(4): p. 329.
645. Hamada, T., M.C. Antle, and R. Silver, *Temporal and spatial expression patterns of canonical clock genes and clock-controlled genes in the suprachiasmatic nucleus.* European Journal of Neuroscience, 2004. **19**(7): p. 1741.
646. Obrietan, K., S. Impey, and D.R. Storm, *Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei.* Nat Neurosci, 1998. **1**(8): p. 693.
647. Butcher, G.Q., et al., *The p42/44 Mitogen-activated Protein Kinase Pathway Couples Photic Input to Circadian Clock Entrainment.* Journal of Biological Chemistry, 2002. **277**(33): p. 29519-29525.
648. Antle, M.C., L.J. Kriegsfeld, and R. Silver, *Signaling within the Master Clock of the Brain: Localized Activation of Mitogen-Activated Protein Kinase by Gastrin-Releasing Peptide.* The Journal of Neuroscience, 2005. **25**(10): p. 2447-2454.
649. Pizzio, G.A., et al., *Nerve growth factor-induced circadian phase shifts and MAP kinase activation in the hamster suprachiasmatic nuclei.* European Journal of Neuroscience, 2005. **22**(3): p. 665.
650. Dziema, H., et al., *The ERK/MAP kinase pathway couples light to immediate-early gene expression in the suprachiasmatic nucleus.* European Journal of Neuroscience, 2003. **17**(8): p. 1617.
651. Hainich, E.C., G.n.A. Pizzio, and D.A. Golombek, *Constitutive activation of the ERK-MAPK pathway in the suprachiasmatic nuclei inhibits circadian resetting.* FEBS Letters, 2006. **580**(28â€“29): p. 6665.
652. Sanada, K., T. Okano, and Y. Fukada, *Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1.* Journal Of Biological Chemistry, 2002. **277**(1): p. 267-271.
653. Whitfield, M.L., et al., *Identification of genes periodically expressed in the human cell cycle and their expression in tumors.* Molecular Biology Of The Cell, 2002. **13**(6): p. 1977-2000.
654. Fambrough, D., et al., *Diverse Signaling Pathways Activated by Growth Factor Receptors Induce Broadly Overlapping, Rather Than Independent, Sets of Genes.* Cell, 1999. **97**(6): p. 727.
655. Iyer, V.R., et al., *The transcriptional program in the response of human fibroblasts to serum.* Science, 1999. **283**(5398): p. 83-87.
656. Israel, M.A., et al., *Probing sporadic and familial Alzheimer/'s disease using induced pluripotent stem cells.* Nature. **advance online publication**.
657. Marchetto, M.C., et al., *Induced pluripotent stem cells (iPSC) and neurologic disease modeling: Progress and Promises.* Human Molecular Genetics.

658. Borel, C., et al., *Identification of cis- and trans- regulatory variation modulating microRNA expression levels in human fibroblasts.* Genome Research. **21**(1): p. 68-73.
659. Shah, S.H., et al., *Association of a Peripheral Blood Metabolic Profile With Coronary Artery Disease and Risk of Subsequent Cardiovascular Events / CLINICAL PERSPECTIVE.* Circulation: Cardiovascular Genetics, 2010. **3**(2): p. 207-214.
660. Wang, T.J., et al., *Metabolite profiles and the risk of developing diabetes.* Nat Med, 2011. **17**(4): p. 448.
661. Monks, S.A., et al., *Genetic inheritance of gene expression in human cell lines.* American Journal Of Human Genetics, 2004. **75**(6): p. 1094-1105.
662. Petretto, E., et al., *Heritability and tissue specificity of expression quantitative trait loci.* Plos Genetics, 2006. **2**(10): p. 1625-1633.
663. Brem, R.B. and L. Kruglyak, *The landscape of genetic complexity across 5,700 gene expression traits in yeast.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(5): p. 1572-1577.
664. DeCook, R., et al., *Genetic Regulation of Gene Expression During Shoot Development in Arabidopsis.* Genetics, 2006. **172**(2): p. 1155-1164.
665. Schadt, E.E., et al., *Genetics of gene expression surveyed in maize, mouse and man.* Nature, 2003. **422**(6929): p. 297.
666. Jin, W., et al., *The contributions of sex, genotype and age to transcriptional variance in Drosophila melanogaster.* Nature Genetics, 2001. **29**(4): p. 389-395.
667. Chesler, E.J., et al., *Complex trait analysis of gene expression uncovers polygenic and pleiotropic networks that modulate nervous system function.* Nature Genetics, 2005. **37**(3): p. 233-242.
668. Bystrykh, L., et al., *Uncovering regulatory pathways that affect hematopoietic stem cell function using 'genetical genomics'.* Nature Genetics, 2005. **37**(3): p. 225-232.
669. Petretto, E., et al., *Integrated gene expression profiling and linkage analysis in the rat.* Mammalian Genome, 2006. **17**(6): p. 480-489.
670. Cheung, V.G., et al., *Mapping determinants of human gene expression by regional and genome-wide association.* Nature, 2005. **437**(7063): p. 1365-1369.
671. Stranger, B.E., et al., *Population genomics of human gene expression.* Nature Genetics, 2007. **39**(10): p. 1217-1224.
672. Michaelson, J.J., S. Loguercio, and A. Beyer, *Detection and interpretation of expression quantitative trait loci (eQTL).* Methods, 2009. **48**(3): p. 265.
673. Altshuler, D., M.J. Daly, and E.S. Lander, *Genetic Mapping in Human Disease.* Science, 2008. **322**(5903): p. 881-888.
674. Stuart, J.M., et al., *A Gene-Coexpression Network for Global Discovery of Conserved Genetic Modules.* Science, 2003. **302**(5643): p. 249-255.
675. Visscher, P.M., R. Thompson, and C.S. Haley, *Confidence Intervals in QTL Mapping by Bootstrapping.* Genetics, 1996. **143**(2): p. 1013-1020.
676. Bing, N. and I. Hoeschele, *Genetical Genomics Analysis of a Yeast Segregant Population for Transcription Network Inference.* Genetics, 2005. **170**(2): p. 533-542.
677. Kulp, D. and M. Jagalur, *Causal inference of regulator-target pairs by gene mapping of expression phenotypes.* BMC Genomics, 2006. **7**(1): p. 125.
678. Tu, Z., et al., *An integrative approach for causal gene identification and gene regulatory pathway inference.* Bioinformatics, 2006. **22**(14): p. e489-e496.

679. Wen, Y., et al., *An eQTL-based method identifies CTTN and ZMAT3 as pemetrexed susceptibility markers.* Human Molecular Genetics.
680. Schadt, E.E., et al., *Mapping the genetic architecture of gene expression in human liver.* Plos Biology, 2008. **6**(5): p. 1020-1032.
681. Goering, H.H.H., et al., *Discovery of expression QTLs using large-scale transcriptional profiling in human lymphocytes.* Nature Genetics, 2007. **39**(10): p. 1208-1216.
682. Bullaughey, K., et al., *Expression quantitative trait loci detected in cell lines are often present in primary tissues.* Human Molecular Genetics, 2009. **18**(22): p. 4296-4303.
683. Nica, A.C., et al., *The Architecture of Gene Regulatory Variation across Multiple Human Tissues: The MuTHER Study.* PLoS Genet. **7**(2): p. e1002003.
684. Ding, J., et al., *Gene Expression in Skin and Lymphoblastoid Cells: Refined Statistical Method Reveals Extensive Overlap in cis-eQTL Signals.* The American Journal of Human Genetics. **87**(6): p. 779.
685. Min, J.L., et al., *The Use of Genome-Wide eQTL Associations in Lymphoblastoid Cell Lines to Identify Novel Genetic Pathways Involved in Complex Traits.* PLoS ONE. **6**(7): p. e22070.
686. Glazier, A.M., J.H. Nadeau, and T.J. Aitman, *Finding Genes That Underlie Complex Traits.* Science, 2002. **298**(5602): p. 2345-2349.
687. Johannessen, M., M.P. Delghandi, and U. Moens, *What turns CREB on?* Cellular Signalling, 2004. **16**(11): p. 1211.
688. Anuforo, D.C., D. Acosta, and R.V. Smith, *Hepatotoxicity Studies with Primary Cultures of Rat Liver Cells.* In Vitro, 1978. **14**(12): p. 981.
689. Guillouzo, A., et al., *Use of human hepatocyte cultures for drug metabolism studies.* Toxicology, 1993. **82**(1â€“3): p. 209.
690. McKim, J.M., Jr., *Building a Tiered Approach to In Vitro Predictive Toxicity Screening: A Focus on Assays with In Vivo Relevance.* Combinatorial Chemistry & High Throughput Screening. **13**(2): p. 188-206.
691. Lee, J.K., et al., *In vitro cytotoxicity tests on cultured human skin fibroblasts to predict skin irritation potential of surfactants.* Toxicology in Vitro, 2000. **14**(4): p. 345.
692. Snyder, E.M. and M.R. Murphy, *Schizophrenia therapy: beyond atypical antipsychotics.* Nat Rev Drug Discov, 2008. **7**(6): p. 471.
693. Margolis, R.L. and C.A. Ross, *Neuronal signaling pathways: genetic insights into the pathophysiology of major mental illness.* Neuropsychopharmacology. **35**(1): p. 350-351.
694. Pagani, L., et al., *Serum factors in older individuals change cellular clock properties.* Proceedings Of The National Academy Of Sciences Of The United States Of America. **108**(17): p. 7218-7223.
695. Mendelsohn, A.R. and J.W. Larrick, *Overcoming the Aging Systemic Milieu to Restore Neural Stem Cell Function.* Rejuvenation Research. **14**(6): p. 681-684.
696. Cavadini, G., et al., *TNF-Î± suppresses the expression of clock genes by interfering with E-box-mediated transcription.* Proceedings of the National Academy of Sciences, 2007. **104**(31): p. 12843-12848.
697. Chari, R., et al., *Integrating the multiple dimensions of genomic and epigenomic landscapes of cancer.* Cancer and Metastasis Reviews. **29**(1): p. 73.
698. Tanaka, E., *Update: genetic polymorphism of drug metabolizing enzymes in humans.* Journal Of Clinical Pharmacy And Therapeutics, 1999. **24**(5): p. 323-329.

699. Gazin, C., et al., *An elaborate pathway required for Ras-mediated epigenetic silencing.* Nature, 2007. **449**(7165): p. 1073.
700. Opavsky, R., et al., *CpG Island Methylation in a Mouse Model of Lymphoma Is Driven by the Genetic Configuration of Tumor Cells.* PLoS Genet, 2007. **3**(9): p. e167.
701. Reya, T. and H. Clevers, *Wnt signalling in stem cells and cancer.* Nature, 2005. **434**(7035): p. 843.
702. Tsang, D.P.F. and A.S.L. Cheng, *Epigenetic regulation of signaling pathways in cancer: Role of the histone methyltransferase EZH2.* Journal Of Gastroenterology And Hepatology. **26**(1): p. 19-27.
703. Santi, D.V., C.E. Garrett, and P.J. Barr, *On the mechanism of inhibition of DNA-cytosine methyltransferases by cytosine analogs.* Cell, 1983. **33**(1): p. 9.
704. Jones, P.A. and S.M. Taylor, *Cellular differentiation, cytidine analogs and DNA methylation.* Cell, 1980. **20**(1): p. 85.
705. Cheng, J.C., et al., *Preferential response of cancer cells to zebularine.* Cancer Cell, 2004. **6**(2): p. 151.
706. Yan, H., et al., *Allelic Variation in Human Gene Expression.* Science, 2002. **297**(5584): p. 1143.

Abbreviations

AANAT	Arylalkylamine N-acetyl transferase
AC	Adenylyl cyclase
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC	β-catenin-Adenomatous Polyposis Coli
ATP	Adenosine-5'-triphosphate
AVP	Arginine vasopressin
BI	Bioluminescence imaging
BMAL1	Brain and muscle ARNT-like protein 1
BMP	Bone Morphogenic Protein
cAMP	Cyclic 3’5’-adenosine monophosphate
CaMK	Ca^{2+}/calmodulin – dependent protein kinase
CLOCK	Circadian locomoter output cycles protein kaput
CREB	cAMP response – element – binding protein
CRE	cAMP responsive element
CRY	Cryptochrome
cGK	Cyclic guanosine monophosphate dependent protein kinase
CKIε	Casein kinase epsilon
DRN	Dorsal raphe nucleus
EEG	Electroencephalography
ERK1/2	Extracellular signal- related kinase
FRD	Free-running disorder
FRQ	Frequency locus
GABA	γ-aminobutyric acid
GC	Gyanylyl cyclase
GHT	Geniculohypothalamic tract
Glu	Glutamate
GR	Glucocorticoid receptor
GRP	Gastrin releasing peptide
GSK-3	Glycogen-Activated Kinase-3
GWA	Genome-wide association studies
HER2	Human Epidermal Growth Factor Receptor 2
HIOMT	Hydroxyindole O-methyltransferase
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
ICER	Inducible cyclic AMP early repressor
IGL	Intergeniculate leaflet
IP_3	Inositol triphosphate receptors
ISWR	Irregular sleep wake rhythm
JNK/ SAPK	Terminal kinase/Stress activated protein kinase
Kir3	Inwardly rectifying potassium channel
LCL	Lymphoblastoid cell line
MAPK	Mitogen –activated protein kinase cascades
MRN	Median raphe nucleus
MSK1	Stress-activated protein Kinase 1

MT1 and MT2	High affinity G – protein coupled melatonin receptors
NF1	Neurofibromin 1
NMDA	N-methyl-D-aspartate
NPAS2	Neuronal PAS domain-containing protein 2
NPY	Neuropeptide Y
nNOS	Nitric oxide synthase
NO	Nitric oxide
OPM	Olivary pretectal nucleus
P	Phosphate groups
PACAP	Pituitary adenylate cyclase-activating peptide
PCK2	Phosphoenolpyruvate carboxykinase 2
PER	Period
PI3K/AKT	Phosphatidylinositol 3-kinase / protein kinase B
PKA	cAMP dependent protein kinase A
PKAII	Cyclic AMP-dependent protein kinase type II
PKC	Protein kinase C
PKG	Protein kinaseG
PLC	Phospholipase C
PRC	Phase Response Curve
PTEN	Phosphatase and tensin homologue
QTLs	Expression quantitative trait loci
RGC	Retinal ganglion cells
RHT	Retinohypothalamic tract
ROS	Reactive oxygen species
RSK	p90 Ribosomal S6 Kinases
RTKs	Receptor tyrosine kinases
SCN	Suprachiasmatic nucleus
SIRT1	Sirtuin
SNP	Single nucleotide polymorphism
SOS	Son Of Sevenless
SP	Substance P
STATs	Signal Transducers and Activators of Transcription
SWD	Shift work disorder
TGF-β	Transforming Growth Factor-β
UCFs	Umbilical cord fibroblasts
VIP	Vasoactive intestinal polypeptide
vLGN	Ventral lateral geniculate nucleus
VPAC2	Vasoactive intestinal peptide receptor 2
5HT	Serotonin

Curriculum Vitae

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EDUCATION

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2004	**Bachelor of Sciences in Chemistry (BSc.)**, Comenius University, Bratislava, Slovakia
1996-2000	**High Medical School**, Trencin, Slovakia

RESEARCH EXPERIENCE

Since 2007	Institute of Pharmacology and Toxicology, University of Zurich, Switzerland **Advisor**: Prof. Steven, A. Brown **Thesis Project**: Profiling inter-individual differences in human cellular signaling
2005 – 2006	**Danube Clone**, Bratislava, Slovakia **Advisor:** Prof. Michal Novak, Peter Filipcik PhD. **Research Project:** Drug development for Alzheimer disease

2002 – 2005	Institute of Biochemistry, Comenius University, Bratislava, Slovakia **Advisor:** Prof. Anton Horvath **Diploma Project:** Down-regulation of the nuclear –encoded subunits of complexes III and IV of the respiratory chain in procyclic *Trypanosoma brucei* by RNAi
2004	Faculty of Parasitology, University of South Bohemia, Ceske Budejovice, Czech Republic **Advisor:** Prof. Julius Lukes **Research Project:** Functional analysis of selected subunits of mitochondrial respiratory complexes in *Kinetoplastida*

PUBLICATIONS

Horvath Anton; Horakova Eva; Dunajcikova Petra; Verner Zdenek; Pravdova Eliska; Slapetova Iveta; **Cuninkova Ludmila**; Lukes Julius (2005). Downregulation of the nuclear-encoded subunits of the complexes III and IV disrupts their respective complexes but not complex I in procyclic *Trypanosoma brucei*. *Molecular Microbiology*; 58, 116-130.

Cuninkova, L. and S.A. Brown (2008). Peripheral Circadian Oscillators, *Annals of the New York Academy of Sciences*, 1129(1):p.358

Cuninkova Ludmila, Maan van der Werken, Ermanno Moriggi, A. Johansson, Gabriella Lundkvist, Marijke Gordijn, and Steven A. Brown (2012) Profiling inter-individual differences in human cellular signaling. *submitted*

TEACHING EXPERIENCE

2008 – 2011	Supervision of students from International Biology Undergraduate Summer School
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2007 **EU Clock Summer School** – 16th European Chronobiology School, Matrahaza, Hungary

ATTENDED MEETINGS

2008 **European Human Genetic Conference**, May 31-June 3Barcelona, Spain

Poster presentation

2008 **The Consortium of European project PROUST: "GENES AT WORK ON TIME"**, October 15-18, Turin , Italy

Poster presentation

2009 **Stromereien**, Performance Festival Zürich,August 29. Juli - 7. August, Zurich Switzerland

Presentation of a Talk

2010 **Annual Meeting, EUCLOCK**, January 25. - 29., Frauenchiemsee, Germany

Talk and Poster presentation

2010 **CHSL/WT Computational Cell Biology**, February 10-13., Cambridge, UK

Presentation of a Talk

2010 **Society for Research on Biological Rhythms Meeting**, May 22-26, Sandestin, FL USA

Poster Presentation - **Society for Research on Biological Rhythms (SRBR) Research Merit Award.**

2011 **Spring Meeting of the Swiss Society of Pharmacology and Toxicology**, April 27 – 28, Zurich, Switzerland

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